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Feasibility of Establishing a Tritrophic Pest Management System in the Greenhouse Industry: An Approach to Utilization of Pathogenic Fungi for Control of Western Flower Thrips, and Pathogenic Fungi With Predators for Synergistic Control of Cabbage Looper.

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial

Fulfillment of

the requirements for the degree of Master of Science

In

Environmental Biology and Ecology

Department of Biological Sciences

Edmonton, Alberta

Fall 2005

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Dedication

This thesis is dedicated to my father, mother, brother and grandparents with love, respect and thanks for always supporting my life goals, and providing enriching life lessons and inspiration to explore and create, whether it involved reverse-engineering or not.

Abstract

Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), and cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), are both serious agricultural and horticultural pests.

Beauveria bassiana (Balsamo) Vuillemin is an important ubiquitous entomopathogen. The main goal of this thesis was to test several isolates of *B. bassiana*, isolated from insects and soil in Alberta, Canada against WFT and cabbage looper including its natural predator *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae).

A whole-insect bioassay system clearly demonstrated the inter- and intra-strain variability in effectiveness inherent to all *B. bassiana* strains tested herein.

Four isolates, 3, 7, 25 and 35 performed better than the commercially available *B. bassiana*-based mycoinsecticide, Emerald BioAgriculture's isolate GHA, when tested against adult female WFT in replicated, comparative, whole-insect bioassays. Isolate 3 also performed better than GHA when tested on cabbage looper, and resulted in a synergistic effect if used in conjunction with *P. maculiventris*. These strains of *B. bassiana* demonstrate commercial potential as biological control agents.

Acknowledgement

I would like to thank my supervisor Dr. B. A. Keddie, professor of insect pathology at the University of Alberta, for supporting my educational aspirations and allowing me to both use and alter his laboratory to meet my research goals. I am also grateful for the specialized statistical and bioassay coaching provided by my co-supervisor Dr. K. M. Fry, former researcher at the ARC Vegreville, Alberta and new professor of science at Olds College, Alberta. I also thank Dr. B. S. Heming, world-class thrips specialist and member of my supervisory committee, for providing superb assistance as I editied my thesis.

To Matthew Greif, Ph.D. graduate student of Dr. R. Currah, for providing several of the isolates of *Beauveria bassiana* tested in this project.

To Rod Werezuk, lab technician at the ARC Vegreville, Alberta, for providing western flower thrips tested in this project and essential support complimenting that of Dr. Fry.

To my research counterpart, M.Sc. graduate student Sunil Rajput, for providing rock-solid, high-quality professional assistance during the course of this project and a means of release from the stresses by discussing their root cause while simultaneously enjoying a beer(s) at the local pub.

To my family, who introduced me to the world of science at a young age and cultivated my mind into a tool which can now help to contribute something of use to the world.

To Jody Gouchie, electrician and long-time friend, who helped me get away from the stresses via adrenaline-fuelled sports and much-needed parties.

I am also grateful for the financial support provided by the NSERC-funded Biocontrol Network of Canada.

Table of Contents

Chapter 1. Literature Review 1
1.1 Introduction 1
1.2 Western Flower Thrips 3
1.3 Cabbage Looper 6
1.4 Alternative Solutions to Insect Pest Management Issues 7
1.4.1 Use of Microbials as an Alternative Means of Insect Pest Control 7
1.4.2 Integrated Pest Management Programs as a Means of Limiting the Negative Impacts of Chemical Insecticide Use 8
1.5 *Beauveria bassiana*, the Entomopathogenic Fungus 10
1.5.1 History of *B. bassiana* 10
1.5.2 Taxonomic Placement of *B. bassiana* 11
1.5.3 Infection Process of *B. bassiana*: Detail of Life History in Insect Host 11
1.5.4 Persistence of *B. bassiana*, and its Natural Epizootics 15
1.6 Factors Affecting Efficacy of *Beauveria bassiana* When Used as a Biological Control Agent 17
1.6.1 Formulations 17
1.6.2 *Beauveria bassiana* Use in Conjunction with Pesticides 17
1.6.3 *Beauveria bassiana* Used in Conjunction with Herbicides and Fungicides 18
1.7 Summary 19
1.8 Figures 20
1.9 Literature Cited 29

Chapter 2. Effects of *Beauveria bassiana* (Balsamo) Vuillemin on Western Flower Thrips, *Frankliniella occidentalis* (Pergande), Using a Leaf-Disc Bioassay 41
2.1 Introduction 41
2.2 Materials and Methods 44
2.2.1 Greenhouse Compartment 44
2.2.2 Bush Beans 45
2.2.3 Propagation of *B. bassiana* 45
2.2.4 Thrips Rearing 46
2.2.5 Buffers 47
2.2.6 Propidium-Iodide Solutions 48
2.2.7 Leaf-Disc Bioassay 49
2.2.7.1 Spray Tower 49
2.2.7.2 Preparation for Bioasay 50
2.2.7.2.1 Viability Assays 50
2.2.7.2.2 Preparation of Conidia and Bioassay Arenas 51
2.2.7.2.3 Preparation of Corrected Conidial Suspensions, and Food Sources for Bioassay Arenas 52
2.2.7.3 Initiation of Bioassay 53
2.2.7.3.1 Bioassay 53
2.2.7.3.2 Observations During Bioassay 54
2.2.8 Statistical Analysis 54
2.3 Results 55
2.3.1 Screening Isolates of *B. bassiana* for Efficacy Against WFT 55
2.3.1.1 Probit Analysis and Analysis of Varience 55
2.3.1.2 Results of the Screening Trials 55
2.3.1.3 Selection of More Virulent Isolates from Screening Trials 57
2.3.2 Evaluation of Selected Strains 57
2.3.2.1 Group 1 of Virulence Evaluation 57
2.3.2.1.1 Performance of Isolates in Group 1 58
2.3.2.2 Group 2 of Virulence Evaluation 59

2.3.2.2.1 Performance of Isolates in Group 2 59
2.4 Discussion 60
2.4.1 *Beauveria bassiana* Screening and Evaluation of Results 60
2.5 Summary 65
2.6 Tables and Figures 67
2.7 Literature Cited 94

Chapter 3. Effects of *Beauveria bassiana* (Balsamo) Vuillemin on Cabbage Looper, *Trichoplusia ni*, and a Predaceous Soldier Bug, Podisus maculiventris, Via Caged-Whole-Plant Bioassay 98
3.1 Introduction 98
3.2 Materials and Methods 103
3.2.1 Greenhouse Compartment 103
3.2.2 Bush Beans 103
3.2.3 Propagation of *B. bassiana* 104
3.2.4 Cabbage Looper Rearing 104
3.2.4.1 Meridic Lepidoptern Diet 105
3.2.5 *Podisus maculiventris* Rearing 105
3.2.6 Buffers 106
3.2.7 Propidium-Iodide Solutions 106
3.2.8 Screening of Selected *B. bassiana* Isolates Against Cabbage Looper . 106
3.2.9 Whole Plant Tritrophic Bioassay 108
3.2.9.1 Hand-Held Sprayers 108
3.2.9.2 Preparation for Bioassay 108
3.2.9.2.1 Solution Preparation, Viability Assays, and Conidial Suspensions 108
3.2.9.2.2 Set-Up of Bioassay Cages 109
3.2.9.2.3 Preparation of Corrected Conidial Suspensions 109
3.2.9.3 Initiation of Bioasay 110
3.2.9.3.1 Bioassay 110
3.2.9.3.2 Observations During Bioassay 110
3.2.10 Statistical Analyses 111
3.3 Results 111
3.3.1 Screening of Selected *B. bassiana* Isolates for Effects Against Cabbage Looper 111
3.3.1.1 Probit Analysis and Analysis of Variance 111
3.3.1.2 Results of Isolate-Selection Trials 111
3.3.2 Evaluation of Selected Strains 112
3.3.2.1 Evaluation of Virulence of *B. bassiana* Isolates for Cabbage - Looper , in Conjunction with Efficacy of Predation on Cabbage - Looper by *P. maculiventris* 112
3.3.2.1.1 Performance of Treatments Administered to Cabbage - Looper 112
3.3.2.2 Evaluation of Virulence of *B. bassiana* Isolates for *Podisus - maculiventris* 113
3.3.2.2.1 Detrimental Effects of Isolates Administered to *Podisus - maculiventris* 113
3.4 Discussion 114
3.4.1 *B. bassiana* Screening and Evaluation of Results 114
3.5 Summary 117
3.6 Tables and Figures 118
3.7 Literature Cited 143

Chapter 4 General Discussion and Conclusions 147
4.1 The Need for Environmentally Friendly Insecticides 147
4.2 Benefits of using Microbial Insecticides Alone or in Conjunction with

Beneficial Insects Instead of Chemical Pesticides 148
4.3 Goals of This Project 150
4.3.1 Control of Western Flower Thrips with Native Alberta *B. bassiana* Isolates 151
4.3.2 Control of Cabbage Looper with Native Alberta *B. bassiana* Isolates, in Conjunction with *P. maculiventris* 151
4.4 The importance of Standardizing Experimental Procedures 152
4.5 The Importance of Screening Isolates Through Replicated Bioassays 152
4.6 The Need to Extend This Research 153
4.6.1 The Biocontrol Network 153
4.6.2 The Pest Management Regulatory Agency 154
4.6.3 Acceptance of Biological Control Agents Worldwide 154
4.7 Further Uses for This Research 155
4.8 Literature Cited 157

Appendices 160

List of Tables

Table 2-1. Performance *B. bassiana* on WFT among screening trials 68
Table 2-2. Cumulative mortality WFT by GHA among scr. trials 69
Table 2-3. Isolates comparable on basis of performance of GHA 70
Table 2-4. Cum. mort. WFT by *B. b.* within scr. trials 71
Table 2-5. Perform. *B. b.* on WFT among evaluation trials 1a - c 73
Table 2-6. Cum. mort. WFT by GHA among eval. trials 74
Table 2-7. Cum. mort. WFT by *B. b.* within pooled eval. trials 75
Table 2-8. Perform. *B. b.* on WFT among eval. trials 2a - c 76
Table 2-9. Cum. mort. WFT by GHA among eval. trials 77
Table 2-10. Cum. mort. WFT by *B. b.* within pooled eval. trials 78
Table 2-11. Viability *B. b.* conidia among scr. trials 79
Table 2-12. Viability *B. b.* conidia among eval. trials 80
Table 2-13. Comparison coverslip counts among scr. trials 81
Table 2-14. Comparison coverslip counts among eval. trials 82
Table 2-15. Origin *B. b.* isolates used in study 83
Table 2-16. Comparison two Alberta isolates tested in thesis and ARC 84

Table 3-1. Perform. *B. b.* on cabbage looper among isolate selection trials 119
Table 3-2. Cum. mort. cab. loop. by *B. b.* within pooled is. sel. trials 120
Table 3-3. Perform. *B. b. P. maculiventris* on cab. loop. among tritrophic trials . 121
Table 3-4. Cum. mort. cab. loop. by *B. b. P. mac.* within pooled trit. trials 122
Table 3-5. Perform. *B. b.* on *P. mac.* among trit. trials 123
Table 3-6. Cum. mort. *P. mac.* by *B. b.* within pooled trit. trials 124
Table 3-7. Comparison cab. loop. damage within pooled trit. trials 125
Table 3-8. Viability *B. b.* conidia among is. sel. trials 126
Table 3-9. Viability *B. b.* conidia among trit. trials 127

Table A1-1. R-square and normality for scr. trials 161
Table A1-2. R-square and normality for eval. trials 162
Table A1-3. R-square and normality for is. sel. trials 163
Table A1-4. R-square and normality for trit. trials 164

List of Figures, Diagrams, and Graphs

Figure 1-1. WFT petal damage campanula 21
Figure 1-2. WFT foliage damage cucumber 22
Figure 1-3. WFT deform damage cucumber 23
Figure 1-4. WFT scar damage cucumber 24
Figure 1-5. WFT life history 25
Figure 1-6. Cabbage looper leaf damage 26
Figure 1-7. Cabbage looper 5th instar 27
Figure 1-8. *Beauveria bassiana* conidiophore 28

Figure 2-1. Greenhouse plant production & watering system 85
Figure 2-2. Greenhouse bush beans & laboratory bush bean chamber 86
Figure 2-3. *Beauveria bassiana* conidia production 87
Figure 2-4. WFT rearing chamber 88
Figure 2-5. Condia spray chamber 89
Figure 2-6. WFT bioassay arena 90
Figure 2-7. WFT cadaver killed by *B. bassiana* 91
Figure 2-8. WFT cadaver on *B. bassiana* selective media 92
Figure 2-9. Correlation coverslip count and % WFT cumulative mortality 93

Figure 3-1. Insect bioasay cage 128
Figure 3-2. Cabbage looper cuticular blistering 129
Figure 3-3. Cabb. loop. cadavers with *B. bassiana* oosporein coloration 130
Figure 3-4. Cabb. loop. cadavers with *B. bassiana* sporulation 131
Figure 3-5. Bush bean damage cabb. loop. with out/with *P. maculiventris* 132
Figure 3-6. Bush bean damage GHA cabb. loop. with out/with *P. mac.* 133
Figure 3-7. Bush bean damage isolate 3 cabb. loop. with out/with *P. mac.* ... 134
Figure 3-8. Cabbage looper acute *B. bassiana* mycosis 135
Figure 3-9. Degrees of bush bean feeding damage by cabb. loop. 136
Figure 3-10. Feeding damage bush beans cabb. loop. 137
Figure 3-11. Feeding damage bush beans cabb. loop. GHA 138
Figure 3-12. Feeding damage bush beans cabb. loop. Isolate 3 139
Figure 3-13. Feeding damage bush beans cabb. loop. *P. mac.* 140
Figure 3-14. Feeding damage bush beans cabb. loop. *P. mac.* & GHA 141
Figure 3-15. Feeding damage bush beans cabb. loop. *P. mac.* & isolate 3 ... 142

List of Abbreviations

For all tables, values presented in scientific notation as nE+d are equivalent to the value n x 10^d.

ARC = Alberta Research Council, Vegreville.

BCN = Biocontrol Network.

B. b. = *Beauveria bassiana*.

B.t. = *Bacillus thuringiensis*.

cab. loop. = cabbage looper.

cum. mort. = cumulative mortality.

EPA = Environmental Protection Agency.

eval. = evaluation.

IPM = integrated pest management system.

is. sel. = isolate selection.

L:D = ratio of light to dark time periods.

M:F = ratio of males to females.

N.D.R. = non-discernable dose response.

N.M. = no mortality resulting from treatment.

PMRA = Pest Management Regulatory Agency.

perform. = performance.

P. mac. = *Podisus maculiventris*.

p.i. = post-innoculation.

RH = relative humidity.

SDAY = Sabouraud dextrose agar plus yeast extract.

scr. = screening.

trit. = tritrophic.

UV = ultra violet.

WFT = western flower thrips.

Chapter 1. Literature Review

1.1 Introduction

To meet the increasing demand for food during the 20th century, the agriculture industry adopted new methods of production to radically increase productivity, a "Green Revolution". The term Green Revolution, coined in the 1960's, refers to the use of specially selected cultivars of crop plants which, if grown in conjunction with pesticides, fertilizers and controlled irrigation, produced far greater yields than traditional cultivars and methods (Hanson *et al.*, 1982; Rosset *et al.*, 2001). Intensive crop production systems use such measures to produce high crop yields in both agricultural and horticultural settings.

Initially, the higher yields proved promising for the new production methods, but with time, use of these methods became deleterious. Replacement of traditional cultivars and production practices with new ones has caused yields to decline in certain areas. For example, in central Luzon, Phillippines, rice yields increased steadily during the 1970's, peaked, and have since declined (Rosset *et al.*, 2001). Environmental costs have been accumulating steadily, ranging from pollution of groundwater to loss and deterioration of ecosystems (Lacey and Goettel, 1995). Eventually, insect populations developed resistance to many pesticides (Lacey and Goettel, 1995). Subsequent pest resurgences and secondary pest outbreaks, the evolution of non-pest insect populations into pest insect populations, have occurred due to elimination of natural enemies, through use of chemical pesticides (Lacey and Goettel, 1995).

Due to misuse, the dominant technology used in today's agriculture is destroying the basis for future production by causing environmental degradation

and spurring outbreaks of agricultural pests; this then results in ever-increasing difficulty and spending to reach production rates comparable to those of the past (Rosset *et al.*, 2001). The constant increase in production costs has bankrupted many smaller producers, and those able to accommodate increasing costs have accumulated more production area to make up for lower per-acre profit (Rosset *et al.*, 2001).

As a result of the usage of chemical pesticides and due to their physical properties, some pesticide residues remain in the environment even though these products are no longer used (Ghandi and Snedker, 1999). These residues may be found on food grown in contaminated soil and in fish living in contaminated water (Ghandi and Snedker, 1999). The U.S. Environmental Protection Agency (EPA) evaluates the results of tests performed on experimental animals, and on plant and animal cells to determine tolerances for pesticides. Residue levels found in food are usually below tolerance levels, but some pesticides have been found in food for which no tolerances have been set. Despite setting specific regulations for tolerable pesticide residues, a small percentage of domestic and imported foods violate the residue tolerances set by the EPA (Ghandi and Snedker, 1999). Regardless of the data provided to the EPA, there are still gaps in the information regarding health effects of pesticides (Ghandi and Snedker, 1999). Increased public pressure and changes in legislation have resulted in the introduction and use of more environmentally responsible agricultural practices. As a result of these changes, the registration of many chemical pestcides has not or will not be renewed (Lacey and Goettel, 1995).

Amidst an uncertain chemical-intensive agricultural industry, there are examples of successful alternative practices. In Cuba, organic farming has enjoyed an unhindered opportunity since the collapse of trade with the former

Soviet Union (Rosset *et al.*, 2001). Unable to import agrochemicals, Cuba faced the worst food crisis of its history. However Cubans were able to feed themselves as well as they had prior to implementation of Green-Revolution style agricultural practices. Cubans eliminated the food crisis by reviving traditional, agricultural practices (Rosset *et al.*, 2001). Cuba is a microcosm of what may occur on a far larger scale if more sustainable agricultural practices are not employed. If current practices are not effective, then we must stop using them, and turn to more sustainable methods of agricultural production and pest control.

1.2 Western Flower Thrips

Western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a major cosmopolitan pest of greenhouse ornamentals and vegetable crops (Pelikan, 1989; Izhevskyi, 1992; Katayama and Tatara, 1994; Gerin *et al.*, 1994; Vacante, 1995; Murphy *et al.*, 1998). Native to Western North America, WFT has spread over vast distances throughout eastern and western Europe, to Israel, Africa, Australia, and Japan (Kropczynska *et al.*, 1988; Mantel and van de Vrie, 1988; Giliomee, 1989; Jenser and Tusnadi, 1989; Lindqvist 1990; Andjus and Vukovic, 1991; Roditakis, 1991; Tsuchiya *et al.*, 1992; Malipatil *et al.*, 1993; Vasiliu, 1993; Martin and Workman, 1994; Gindin *et al.*, 1996; Vierbergen, 1996).

Western flower thrips is found primarily in flowers, where immatures and adults obtain pollen contents, nectar, and plant fluids via use of piercing-sucking mouthparts, for growth and reproduction, damaging plant tissues as they feed (Figure 1-1) (Higgins, 1992). The mouthparts are unique among insects in that they are asymmetric, consisting of only the left mandible (the right mandible is

vestigial) which is closed apically and fused to the exoskeleton, a pair of interlocking maxillary stylets which are open apically to form a feeding tube, and an extrudable labral pad which is fringed by numerous small papillae (Chrisholm and Lewis, 1984; Hunter and Ullman, 1989). The mouthparts are contained within a compact mouth cone. Feeding is accomplished by piercing plant cells with the mandible, followed by insertion of the maxillary stylets, and removal of cell contents through the feeding tube formed by the maxillae (Chrisholm and Lewis, 1984; Hunter and Ullman, 1989). The labral pad may be extended to facilitate removal of surface fluids sucked through the maxillary stylets (Chrisholm and Lewis, 1984; Hunter and Ullman, 1989). The small papillae on the labral pad may help prevent loss of plant fluids while feeding. The paraglossae are innervated, with 10 pairs of sensory cones in 3 varieties; the precibarial canal is also innervated, containing gustatory sensilla (Hunter and Ullman, 1989). The sensory structures have been implicated in helping the thrips find suitable hosts for feeding (Hunter and Ullman, 1989).

Plants quickly lose water through areas of epidermis damaged by WFT feeding. If plants survive attack, areas that continue to develop often become distorted, the most common result being curled leaves and fruits (Figure 1-3) (Lewis, 1973). On fully developed leaves, light infestations result in silvering and scarring in patches on the laminae (Figure 1-2) or streaks along the veins; heavy infestations on leaves result in drying, withering, and then shedding (Lewis, 1973). Fruitlets attacked early in development are fed on around the calyx or under the sepals, resulting in a ring-shaped scar around the fruit (Lewis, 1973). As the fruit grows, the epidermal scars remain their initial size, which results in deformed fruit (Figure 1-3), as though a tight girdle was secured to the scarred areas; older fruits that are attacked develop cracked scars (Figure 1-4) (Lewis, 1973).

Several disease-causing viruses may be transmitted by WFT through their piercing-sucking fluid feeding and pollen feeding habits. The tomato spotted wilt tospovirus and impatiens necrotic spot tospovirus, both of the family Bunyaviridae, are transmitted through the piercing-sucking mode of feeding (Ullman *et al.* 1997). Recently WFT has been implicated in the transmission of pelargonium flower break carmovirus, prune dwarf ilavirus, and prunus necrotic ringspot ilavirus, through contamination of the body with macerated virus-infected pollen particles and subsequent contamination of wounds caused by the piercing-sucking mode of feeding (Krczal *et al.*, 1995; Ullman *et al.* 1997). Together, these viruses infect several different plant species (Reed and Sukamto, 1995; van de Wetering *et al.*, 1996 and 1999; Wijkamp *et al.*, 1995).

Both females and males account for feeding damage to plants, with females causing oviposition damage as well. Females predominate as they often live longer than the males: the average longevity of a female at 20°C, under controlled conditions, may span 56.8 days from egg to death of adult (Lewis, 1973 and 1997). Males and females mate within an average of 2.4 days of adult eclosion, at 20°C (Lewis, 1997). Eggs are large in relation to the female's abdomen, thus only a few are ready for oviposition at a time (Lewis, 1973). A female WFT may produce 130 to 230 eggs during her lifetime (Lewis, 1973; McDonough *et al.*, 2002). The eggs produced by WFT are cylindrical, slightly kidney-shaped with a white to yellow shell (Figure 1-5). Females deposit eggs directly into leaves, sepals or petals through an incision made by the ovipositor, damaging these plant tissues in the process (McDonough *et al.*, 2002). At 20°C, embryogenesis requires an average of 6.4 days (Lewis, 1997). There are 6 instars in WFT (Figure 1-5); with development intermediate between holometabolous and hemimetabolous (Lewis, 1973; Heming, 1991). The first two larval instars are

able to feed, and account for damage to plant tissues. At 20°C, the first and second instar larvae require average developmental times of 2.3 and 5.2 days respectively (Lewis, 1997). After the second moult, a propupa emerges which is a discrete instar, intermediate between a larva and a pupa (Heming, 2003). The propupa has wing buds and is able to walk slowly, but lacks functional mouthparts and is not able to feed or excrete (Lewis, 1973; Heming, 1993). After the third moult, the pupa emerges, which is naked and quiescent (Lewis, 1973). At 20°C, the propupa and pupa require average developmental times of 2.2 and 2.9 days respectively. After the fourth moult the adult emerges, mates, and the cycle resumes. The average developmental cycle from egg to pre-oviposition adult, at 20°C, is 21.4 days (Lewis, 1997).

1.3 Cabbage Looper

Cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) is a widespread, polyphytophagous noctuid (Vail *et al.* 1971). This pest has been found in environments ranging from North America to the UK, Turkey, and eastward to India and Japan (Coster, 1980; Brown, 1982; Kirby, 1982; Giray, 1985; Chamberlin and Kok, 1986; Lasota and Kok, 1989; Knill-Jones, 1994; Harit and Harit, 2002; Nasu *et al.*, 2003).

Cabbage looper has been reported to feed on approximately 160 species of plants in 36 families (Sutherland and Greene, 1984). They attack lettuce, beans (Figure 1-6), and ornamental plants in the greenhouse cropping system (Sutherland and Greene, 1984). Weeds have been demonstrated to be important hosts for this insect in Israel and the south-western USA (Sutherland and Greene, 1984).

Females predominate, with a sex ratio of 1:1.5 M:F (Gaikwad *et al.*, 1980). Adult moths rest on the underside of host leaves, in debris below host plants, in vegetation bordering the host field or in other protected areas (Mitchell and Chalfant, 1984). Copulation occurs between 1:00AM and 3:30AM (Mitchell and Chalfant, 1984). Adults feed and oviposit one-half hour before sunset (Mitchell and Chalfant, 1984). Eggs are deposited by adult females on the leaves of host plants (Gaikwad *et al.*, 1980). At 25±1°C, under controlled conditions, the egg period ranges from 3 to 4 days (Gaikwad *et al.*, 1980). The larval period ranges from 9 to 17 days, and the larvae pass through five instars based on head capsule size (Gaikwad *et al.*, 1980). Maximum weight gain (up to 68.07%) occurs during the fifth instar (Figure 1-7)(Gaikwad *et al.*, 1980). Prepupation begins at 9 to 17 days after hatch, and is indicated by a lighter, uniform body color and cocoon-spinning, which lasts for 1 day (Gaikwad *et al.*, 1980). Pupation begins at 11 to 19 days, and lasts for 6 to 9 days (Gaikwad *et al.*, 1980). Adults emerge over approximately 3 days and typically survive 6 to 9 days, the total life cycle requiring approximately 24 days (Gaikwad *et al.*, 1980).

1.4 Alternative Solutions to Insect Pest Management Issues

1.4.1 Use of Microbials as an Alternative Means of Insect Pest Control

Microbial control of pest insects is increasing in importance because of increasing resistance of arthropods to chemical insecticides, improved performance and cost-competitiveness of microbial insecticides, reduced environmental costs, and a decrease in development of new chemical insecticides (Starnes *et al.*, 1993); by 1993, use of chemical pesticides was

growing at a rate of only 1 to 2% per year. As a result of stagnation in the global agrochemical market in 1993, the industry turned to export markets, and expanded into South America, Asia and the Middle East (Dinham *et al.* 2005). In 1997, a 1.3% increase in the global agrochemical market occurred (Dinham *et al.* 2005), and in 1998, a 0.9% increase (Dinham *et al.* 2005). Since 1999, global agrochemical sales have decreased by 3.1% and continue to decrease (Dinham *et al.* 2005). Microbial insecticides accounted for only 1% of the insecticide market in 1993, yet use of microbials at that time (mainly *Bacillus thuringiensis, B.t.*) was growing at a rate of 10 to 25% per year (Starnes *et al.*, 1993).

1.4.2 Integrated Pest Management Programs as a Means of Limiting the Negative Impacts of Chemical Insecticide Use

With calls for reduced use of chemical insecticides, but a constant need for insect pest control, integrated pest management programs have been developed (Barbosa and Segarra-Carmona, 1993). Natural enemies of pest insects may be used as integral components of integrated pest management programs (Stern *et al.*, 1959). Such natural enemies include predators, parasitoids, and entomopathogenic bacteria, fungi, and viruses (Lacey and Goettel, 1995).

Integrated pest management can be defined as "a strategy of pest containment that seeks to maximize the effectiveness of biological and cultural control factors, using chemical controls only as needed and with a minimum environmental disturbance" (Luna and House, 1990; Stern *et al.*, 1959). Entomopathogenic bacteria, fungi and viruses are ideal for integrated pest management programs because they are relatively safe to use and have a

narrower spectrum of activity than chemical insecticides (Lacey and Goettel, 1995). Potential antagonistic effects can be limited through careful selection of entomopathogens which have a greater effect on pest insects than on their predators or parasitoids (Luna and House, 1990; Lacey and Goettel, 1995).

Effective microbial control of pest insects often requires the inundative use of entomopathogenic organisms (McCoy *et al.*, 1988). Use of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin as an inundative control agent began in the late 1800's, but interest in this approach diminished with the advent of chemical pesticides (Lacey and Goettel, 1995). Interest was revived in the 1960's with the appearance of several products for use on various pests in The People's Republic of China, and for control of Colorado potato beetle, *Leptinotarsa decimlineata* Say (Coleoptera: Chrysomelidae) in the former Soviet Union (Lacey and Goettel, 1995).

Control of susceptible insect hosts with *B. bassiana* requires optimum formulation of inoculum and application to the affected area at the right time (Barbosa and Segarra-Carmona, 1993). Numerous biotic and abiotic factors affect the efficacy of *B. bassiana* as a biological control agent and must be considered while researching formulations. Such factors for *B. bassiana* include sunlight (UV), desiccation, temperature, humidity, vigour and age of conidia, inoculum thresholds, pesticide contamination, and antagonistic microbes; host behaviour, physiological condition and age are also important (Lacey and Goettel, 1995).

Attempts to increase the efficacy of *B. bassiana* and other entomopathogenic fungi, via genetic manipulation, have lagged behind those for viruses and bacteria. Goals for manipulation of fungal genomes include

increased virulence and resistance to negative environmental factors and fungicides (Lacey and Goettel, 1995).

Caution must be exercised in using microbial control agents. Microbes such as *B.t.* produce a toxic protein which requires a specific site of action to kill insects. If such biological control agents were simply used as replacements for chemicals, i.e., inserting a *B.t.* toxin gene into the genome of an agricultural crop, they would surely encounter similar problems to those faced by chemical pest control methods (Lacey and Goettel, 1995). Intact microbial entomopathogens such as *B.t.* and *B. bassiana* may not experience the potential problems faced by chemical insecticides and genetically-modified-organisms (GMO's) as they use a more dynamic system for infecting and killing host insects, including enzymes functioning in invasion and production of toxic metabolites; these processes can co-evolve with the host insect, whereas a non-living toxin cannot. Thus intact microbial organisms such as *B.t.* and *B. bassiana* are sustainable in integrated pest management programs through their dynamic relationship with insects. The word "integrated" must be the continual focus of integrated pest management programs to ensure their successful and long-term use, within the environment and with other pest management tools (Lacey and Goettel, 1995).

1.5 *Beauveria bassiana*, the Entomopathogenic Fungus

1.5.1 History of *B. bassiana*

Beauveria bassiana is named after Agostino Bassi, born September 23, 1773. Bassi studied law, then pursued agricultural and scientific research until his death in 1856. In 1807, Bassi investigated the muscardine disease of silkworms, *Bombyx mori* (L.), and after several fruitless attempts punctuated by recurring

illness, discovered the "extraneous germ", a parasitic fungus, via a series of elegant experiments, and published his results in 1835, in the monograph "Del Mal del Segno Calcinaccio o Moscardino". Bassi was the first to elucidate the etiology of a contagious microbial disease and to effect the experimental infection of one living organism by another (Ainsworth, 1956).

1.5.2 Taxonomic Placement of *B. bassiana*

Beauveria bassiana is currently placed within the phylum Deuteromycotina (Fungi Imperfecti), class Hyphomycetes, within which there is no formal classification of families (Ainsworth *et al.*, 1983). Fungi placed within this phylum have no known sexual stage, although they may have mechanisms for exchanging genetic material (Ainsworth *et al.*, 1983). A *Cordyceps* sexual stage has recently been discovered in the People's Replublic of China, *Cordyceps bassiana* (Li *et al.*, 2001). The association of the sexual with the asexual stage now allows for the placement of this fungus within the phylum Ascomycotina, class Pyrenomycetes, order Hypocreales, family Clavicipitaceae (Rehner *et al.*, 2005). Regardless of the discovery of a sexual stage, most reproduction is asexual, whereby asexual propagules called conidia (Figure 1-8) are produced (Ainsworth *et al.*, 1983).

1.5.3 Infection Process of *B. bassiana*: Detail of Life History in Insect Host

Experiments show that *B. bassiana* requires a carbon-energy source regardless of the medium on which the fungus is grown (Smith and Grula, 1981). Conidia have sufficient endogenous nitrogen reserves to germinate but

additional exogenous nitrogen sources are required for further growth and extension of hyphae, otherwise autolysis will occur (Smith and Grula, 1981). A wide variety of compounds in the host insect body support luxuriant growth; such compounds include glucose, N-acetylglucosamine, glucosamine, several amino acids, chitin, starch and low levels of certain fatty acids (Smith and Grula, 1981). Two groups of amino acids best support germination and hyphal growth; one includes alanine, valine and leucine (non-polar side-chain), and the other, phenylalanine, tryptophan (non-polar side-chain) and tyrosine (polar, uncharged side-chain) (Smith and Grula, 1981).

For *B. bassiana* to successfully infect an insect host, a sufficient number of conidia must adhere to its integument (Pekrul and Grula, 1979). If the dosage is large, conidia adhere to all body regions, and may clump. Conidia germinate on all regions of the cuticle, and the growing germ tubes show a positive chemotaxis toward cuticle; thus it is possible for several infections to occur simultaneously (Pekrul and Grula, 1979). During cuticle penetration, fungal enzymes are secreted in the immediate vicinity of the hyphal tip. Penetration occurs without formation of specialized apressorial structures, though some germ tubes may show thickenings (Pekrul and Grula, 1979). Penetration may occur as early as 16 - 18 hr post inoculation (p.i.) for highly pathogenic strains (Pekrul and Grula, 1979). The germ tube creates a clean hole at the point of penetration. The fungus may also enter the respiratory system via conidial contamination and germination, or via germ tube penetration through the opening or side of the spiracle (Pekrul and Grula, 1979). By 48 hr p.i., extensive fungal growth may be present, with minimal tissue damage. At 72 hr p.i., the first tissue to show degradation is the fat body followed by the gut tissue and Malpighian tubules (Pekrul and Grula, 1979). At 4 to 6 days p.i., death and mummification have

occurred, with the hemocoel completely filled with fungal growth (Pekrul and Grula, 1979). Hyphal re-emergence occurs when a relative humidity of at least 70% is reached (MacLeod *et al.*, 1966). Hyphae exit first at intersegmental regions followed by appearance of fungi all over the body (Pekrul and Grula, 1979).

Beauveria bassiana may also infiltrate the host insect though the walls of the alimentary tract; this mode of entry has additional effects on the infected insect (Broome *et al.*, 1976). Larvae of the imported fire ant, *Solenopsis richteri* (Forel) (Hymenoptera: Formicidae) perorally infected and then surface sterilized by a bleach solution, experienced 79% mortality (Broome *et al.*, 1976). Larval guts showed a change in pH followed by hyphal penetration, which corresponded to a change in hemolymph pH (Broome *et al.*, 1976).

After infection with *B. bassiana*, a rapid decline in hemolymph components including proteins, amino acids, and minor sugars occurs, with increase in total basic amino acids and glucose (Cheung and Grula, 1982). The physical presence of fungus and toxin production in the alimentary canal may lead to malfunction of the digestive tract; feeding ceases, which likely indicates difficulty processing food after infection (Cheung and Grula, 1982). Resulting hemolymph changes may also result from starvation (Cheung and Grula, 1982). Starvation is not the ultimate cause of death but rather stress caused by infection, depletion of hemolymph components, and the presence of toxic metabolites, combine in lethal synergism to contribute to terminal mycosis (Cheung and Grula, 1982).

Beauveria species produce a suite of entomotoxic metabolites, one of which is beauvericin. Beauvericin belongs to a group of depsipeptide metabolites found in Deuteromycota (Fungi Imperfecti), including *Beauveria*, *Tolypocladium*, *Metarrhizium*, and *Fusarium* species (Žižka and Weiser, 1993).

Among different organisms, beauvericin may affect different tissues in different ways. It can be isolated and purified to a crystalline material and will kill larvae of the mosquito *Culex pipiens* L. (Diptera: Culicidae), at a concentration of 0.1 mg beauvericin per ml water (Žižka and Weiser, 1993). After treatment, 44% of larvae were immobilized and sank, yet still had pulsating dorsal vessels; these intoxicated larvae died after 48 hours, without mortality in remaining larvae (Žižka and Weiser, 1993). Initial intoxication produced small spots of affected cells, and mortality resulted from destruction of tissues (Žižka and Weiser, 1993). Tissues throughout the larva were affected, with differing degrees of destruction, vacuolization, and reduced density of cytoplasm (Žižka and Weiser, 1993). Mitochondria within fat body cells were vacuolized and glandular cells were similarly, but less affected; muscles became disorganized. The midgut was heavily damaged by intensive vacuolization of epithelial cells, progressive degeneration of nuclei, and reduction of microvilli. No melanized spots appeared in the central part of the midgut (Žižka and Weiser, 1993). Beauvericin caused the most intense destruction of muscles and the labyrinth and basal membrane of the midgut, in comparison to other insecticidal fungal metabolites (Žižka and Weiser, 1993). Beauvericin had acute toxic effects, however only if a large dosage was applied (0.1mg/mL), therefore this metabolite was considered only weakly toxic (Žižka and Weiser, 1993). Descriptions of the impacts of beauvericin on WFT or cabbage looper tissues are not available.

After *B. bassiana* kills its insect host, the cadaver may turn red due to production of oosporein, a potent dibenzoquinone (Eyal *et al.*, 1994). This mycotoxin was first isolated from *Oospora colorans* van Beyma (possibly misidentified, Currah, pers. comm.) in 1944 by Kogl and van Wessem (Taniguchi *et al.*, 1984). *Oospora colorans* is an anamorph of the ascomycete *Erysiphe* sp.,

which includes powdery mildew (*Oospora* sp. is an obsolete synonym for *Oidium* sp.). Tested on rat liver mitochondria, oosporein had an effect on the respiratory chain enzymes, although it acted neither as an uncoupler nor phosphorylation inhibitor (Taniguchi *et al.*, 1984). This mycotoxin is not produced in all strains of *B. bassiana*, but when present may act as a significant virulence factor, having its greatest effect after hyphal penetration of the host insect (Rajput, personal communication; Eyal *et al.*, 1994). At this point during the infection process, oosporein may act against intestinal flora as an antagonist (Eyal *et al.*, 1994). Oosporein may also affect antagonistic fungi or bacteria present in soil, which may help *B. bassiana* persist while it is growing in soil as a saprotroph (Eyal *et al.*, 1994). This metabolite can also serve as an indicator of biological control in integrated pest management programs which use *B. bassiana* (Eyal *et al.*, 1994).

Bacterial septicemia may also result from infection with *B. bassiana*, if fungal hyphae are in the process of invasive action during larval moulting. The new integument may be weakened, resulting in lesions through which bacteria can invade (Poprawski *et al.*, 1997).

1.5.4 Persistence of *B. bassiana*, and its Natural Epizootics

Epizootics caused by entomopathogenic fungi may occur sporadically in insect populations with weather, climate, and host susceptibility acting as important determining factors (MacLeod *et al.*, 1966). Fungi grow best in damp habitats where water content of the substrate and atmosphere is high. Conidia of *B. bassiana* require at least 70% relative humidity in the immediate microhabitat to germinate (MacLeod *et al.*, 1966). Unusually high host population density favours development and spread of fungal epizootics. However, fungi

such as *B. bassiana* are capable of initiating epizootics at relatively low host population densities, because the conidia can be dispersed widely, thus affecting the next host generation even though it is still at a low density (Macleod *et al.*, 1966).

Conidia becoming airborne depend on external forces to deliver them to their hosts. If conidia are liberated high above the ground they can be distributed randomly by air currents, and may be carried significant distances from their source (MacLeod *et al.*, 1966). If conidia are liberated close to the ground, they may not travel far. The rate at which conidia settle depends on their density, volume, surface area exposed to air, shape, and surrounding atmospheric humidity, as they will absorb water from a humid atmosphere and become heavier, thus settling faster (Macleod *et al.*, 1966). Rain-washing of air may abruptly end dispersal of conidia (MacLeod *et al.*, 1966).

Conidia may lose viability due to water loss, or by exposure to sunlight (UV). In an insect cadaver, *B. bassiana* can persist as a compact mass of vegetative hyphae, a pseudosclerotium during winter months, and during the following spring, may produce conidia-bearing cells (MacLeod *et al.*, 1966). Cadavers of insects impregnated with such pseudosclerotial material may be found under bark or anchored to a substrate such as a branch, but such cadavers usually disintegrate and the material is disseminated by wind and rain, to come to rest in the soil. Here, the less fastidious fungi, including many of the deuteromycota, enter a saprobic phase or a passive state; it is not known how long *B. bassiana* is able to persist while in such a state (Macleod *et al.*, 1966).

Several biotic and abiotic factors favouring fungal epizootics must be in place for a localized outbreak to expand to epizootic proportions. The fungus must be virulent and the host susceptible and in sufficient density to maintain the

infection chain constituting the epizootic (Macleod *et al.*, 1966). These conditions are readily available in environments provided by greenhouse cropping systems.

1.6 Factors Affecting Efficacy of *B. bassiana* When Used as a Biological Control Agent

1.6.1 Formulations

Conidia of *B. bassiana* have been included in a wide variety of formulations to facilitate delivery to pest-infested cropping systems, and/or contamination of pest insects. Many field trials using various formulations of Emerald BioAgriculture's isolate GHA, proved to be highly successful at controlling various species of grasshoppers/locusts in North America and Africa (Jaronski and Goettel 1997). Formulations used in trials include oil-based, clay-based, wheat-bran, and emulsifiable suspensions (Jaronski and Goettel 1997). In addition, strain selection is also an important factor to consider when dealing with different pest insects and limiting environmental constraints (Jaronski and Goettel 1997).

1.6.2 *Beauveria bassiana* Use in Conjunction with Pesticides

There are instances where the efficacy of *B. bassiana* has been increased by simultaneous use of other chemicals typically used by greenhouse growers. *Beauveria bassiana* kills adults and nymphs of the Tarnished Plant Bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) on cotton (Steinkraus and Tugwell, 1997). Conidia remained infective for up to 96 hours on cotton plants. Infectivity decreased over time, and in addition, more time was required for *B.*

bassiana to kill insects than chemical pesticides (Steinkraus and Tugwell 1997). Better results were attained in the field where a commercially available *B. bassiana* product was used in conjunction with imidacloprid (Steinkraus and Tugwell 1997). The *B. bassiana* product alone was more effective than imidacloprid alone. However imidacloprid worked faster, therefore a combination of the two resulted in a synergistic increase in efficacy (Steinkraus and Tugwell 1997).

Emerald BioAgriculture's isolate GHA, used with Dimilin at only 10% of the recommended field application rate, resulted in a synergistic improvement of field performance, for control of grasshoppers, *Melanoplus sanguinipes* (Fab.) (Jaronski and Goettel 1997).

1.6.3 *Beauveria bassiana* Used in Conjunction with Herbicides or Fungicides

Several chemicals having an antagonistic effect on *B. bassiana* are also readily used in the greenhouse cropping system. *In-vitro* treatments with fungicides such as Chlorothalonil, Maneb, Thiophanate-Methyl, Mancozeb, Metalaxyl+Mancozeb, and Diquat all significantly reduced *B. bassiana* mycelial growth (Todorova *et al.*, 1998). The herbicide Glufosinate-Ammonium significantly reduced *B. bassiana* mycelial growth (Todorova *et al.*, 1998). These chemicals should not be used with *B. bassiana*, especially in cases where chemical insecticides cannot be used.

1.7 Summary

Beauveria bassiana is a naturally occurring fungus, which periodically causes epizootics within and amongst insect populations. Its potential as a biological control agent has been recognized for decades. This fungus can be mass produced and marketed to greenhouse growers in a variety of formulations. *Beauveria bassiana* is dynamic, and attacks insects by a variety of mechanisms, each of which evolves with insect populations, and characterizes a particular strain's virulence. There is potential far beyond current efforts in Canada to develop this fungus for control of insect pests as part of integrated pest management programs.

1.8 Figures

Figure 1-1. Petals of *Campanula* sp. damaged by WFT feeding. Courtesy of Dr. K. Fry.

Figure 1-2. Cucumber foliage damaged by WFT feeding. Courtesy of Dr. K. Fry.

Figure 1-3. Cucumber fruit deformed by WFT feeding damage. Courtesy of Dr. K Fry.

Figure 1-4. Mature cucumber fruit scarred at WFT feeding sites. Courtesy of Dr. K. Fry.

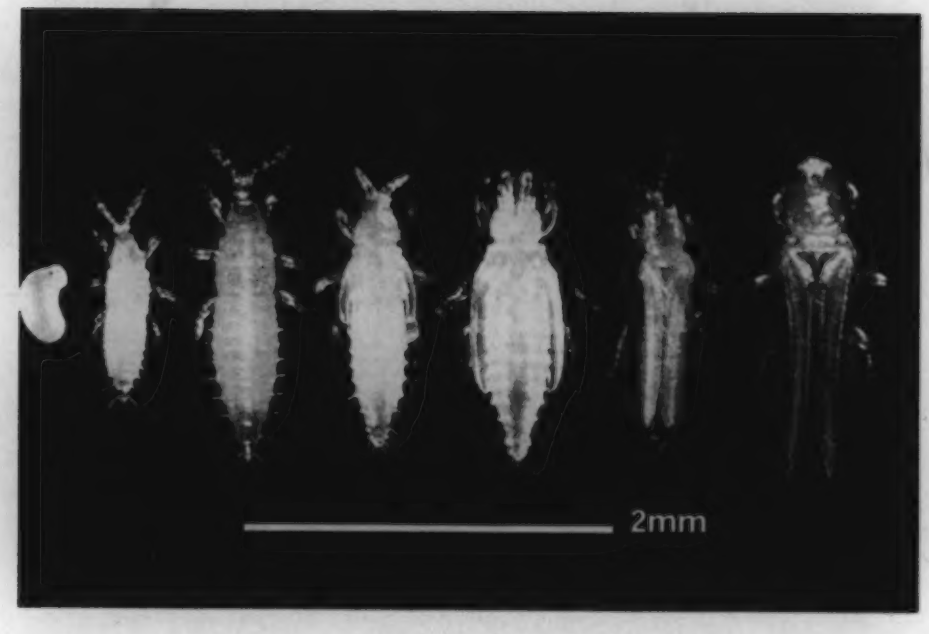

Figure 1-5. The developmental stages of western flower thrips. From left to right: egg (at red-eye stage), first instar larva, second instar larva, propupa, pupa, adult male, adult female. (© CAB International 1997, Thrips as Crop Pests.)

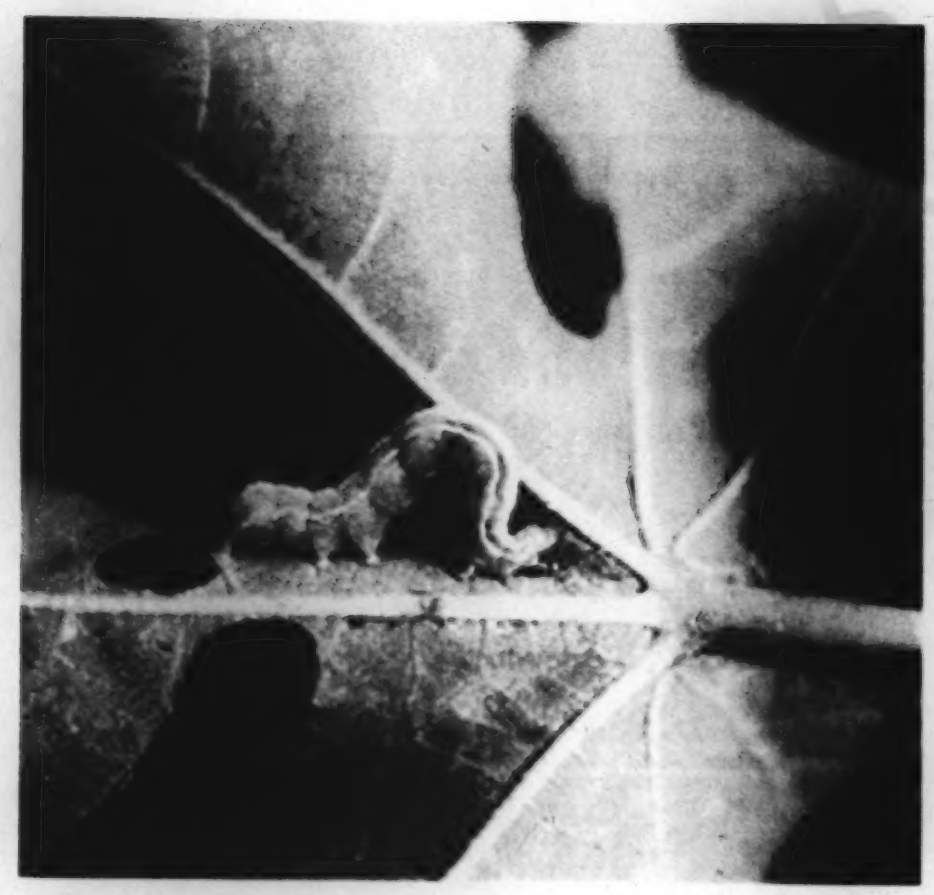

Figure 1-6. Damage to plant leaves at cabbage looper feeding sites.

(Copyright © 2003 University of Tennessee, Institute of Agriculture.)

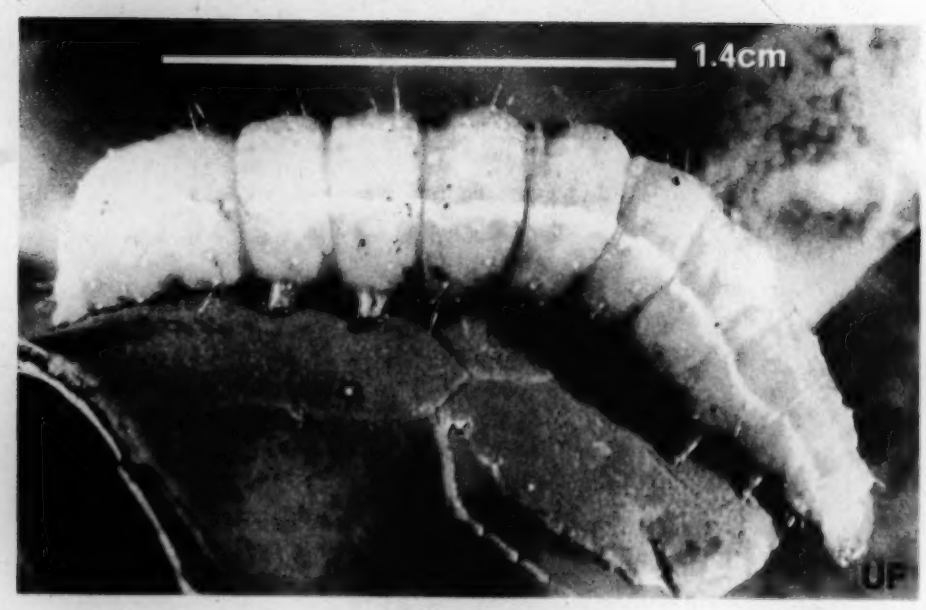

Figure 1-7. Cabbage looper, 5th instar. (Copyright 2001 John L. Capinera, University of Florida.)

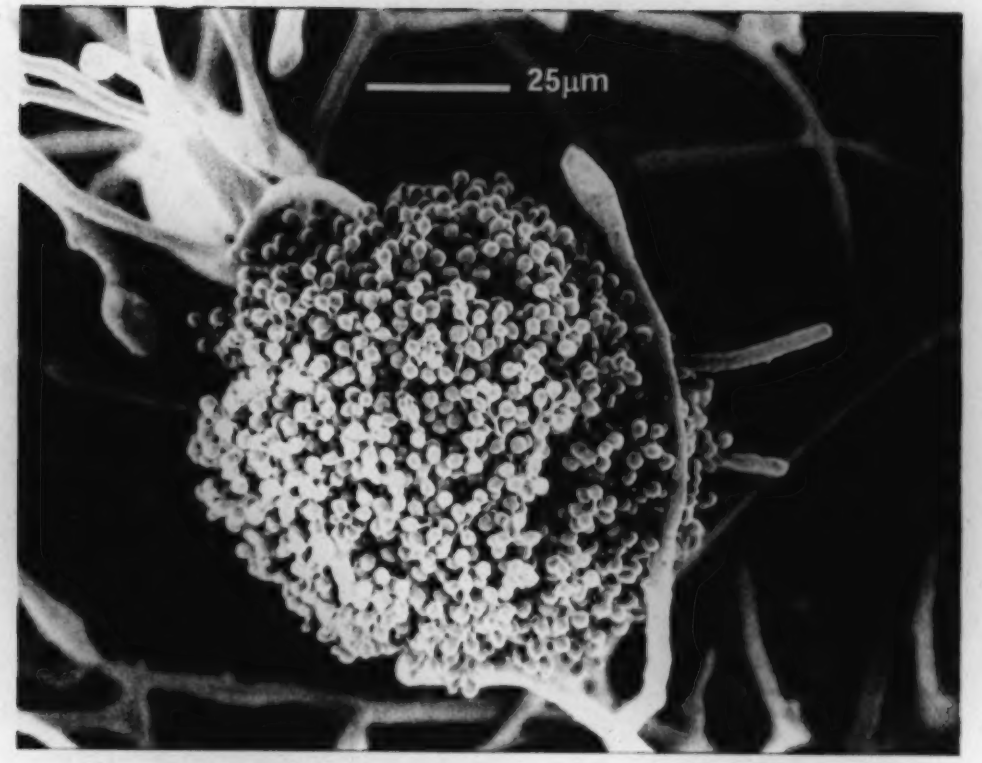

Figure 1-8. *Beauveria bassiana* conidiophore. Courtesy of Dr. B. A. Keddie

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Chapter 2. Effects of *Beauveria bassiana* (Balsamo) Vuillemin on Western Flower Thrips, (WFT) *Frankliniella occidentalis* (Pergande), Using a Leaf-Disc Bioassay.

2.1 Introduction

Initially, western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) was easily controlled by a few applications of insecticide but over time many populations developed resistance (Higgins, 1992). The development of insecticide resistance among these insects is facilitated by their short generation time and numerous progeny. The reproductive mode employed by WFT also lends itself to development of pesticide resistance as they are both parthenogenetic and facultatively arrhenotokous, through which females produce males where there are none, mate with these males, and thereby prevent loss of resistance genes (Robb *et al.* 1995). The spread of pesticide-resistant WFT throughout the greenhouse cropping industry has been facilitated by frequent pesticide application, migration of WFT between regions of agricultural production, and international transport of infested plants (Robb *et al.* 1995).

Biological control of WFT, using predaceous arthropods, has numerous drawbacks. Predaceous mites such as *Neoseiulus (Amblyseius) cucumeris* (Oudemans) (Acari: Phytoseiidae) are unreliable (Higgins, 1992), and substantial numbers of this mite must be maintained for successful control of WFT. Predatory mites are often difficult to establish in the greenhouse, are only able to attack first and early second instar WFT, and their predatory activity is easily inhibited by plant hairs (Shipp and Whitfield, 1991; Higgins, 1992). Larger predators such as minute pirate bugs, *Orius tristicolor* (White) (Hemiptera: Anthocoridae), are more

reliable than *N. cucumeris*, because they attack all life stages of WFT and are not known to be inhibited by plant hairs (Higgins, 1992). However, *Orius tristicolor* is expensive to maintain at an effective density, and may not arrive in a state providing effective control of WFT (e.g., only 30±10% viable when received after four orders of this product, data not shown). In addition, this predator may be difficult to establish in the greenhouse due to cannibalism, photoperiod-induced diapause, and inadequate alternate food sources (Tavella *et al.*, 2000; Askari and Stern, 1972).

Other biological control agents including entomopathogenic fungi have been observed in the field and laboratory (Butt and Brownbridge, 1997). *Verticillium lecanii* (Zimm.) Viagas, a deuteromycete, has been shown to infect and kill WFT. This fungus is an effective suppressor of WFT (Moritz and Frolich, 1996). Conidia germinated 6 hours post innoculation (p.i.), and at 12 hours p.i. the host displayed pathological symptoms and was covered by a network of hyphae. The hyphae exuded sticky mucilage which stuck the host to the substrate (Moritz and Frolich, 1996). Cuticle-degrading enzymes secreted at the tips of unspecialized hyphae allow penetration. Death resulted from multiple lesions on the cuticle caused by cuticle-degrading enzymes in the exuded mucilage, in addition to mycotoxin secretion. Few or no hyphae were present in the hemocoel at the time of death. After death, the hemocoel was colonized by septate filaments, followed by formation of blastospores (Schreiter *et al.* 1994).

Metarhizium anisopliae (Metschnikoff) Sorokin, also a deuteromycete, has also been shown to infect and kill WFT. Its conidia adhered to host cuticle through hydrophobic interactions as well as by secretion of mucilage. Adhesion, germination and differentiation of hyphae into appressoria and penetration pegs all occurred readily on the cuticle of WFT. Penetration of the cuticle occurred

through production of cuticle-degrading enzymes and mechanical pressure (Vestergaard *et al.* 1999). Death occurred with little or no fungal material present in the hemocoel and probably resulted from production of mycotoxins (Vestergaard *et al.* 1999). After death, the hemocoel was colonized by short hyphal bodies (Vestergaard *et al.* 1999; Gerritsen and Weigers, 2000).

An ascomycete (previously classified as a deuteromycete), *Beauveria bassiana* (Balsamo) Vuillemin has been recovered from WFT (Butt and Brownbridge, 1997), but the WFT-specific infection process and pathological effects of this fungus have been little studied (Butt and Brownbridge, 1997). Several comparative bioassays have been performed to identify isolates of entomopathogenic fungi, including *B. bassiana*, which effectively control WFT. One confirmed that pathological effects are greatest for WFT treated with *M. anisopliae*, followed by *B. bassiana* and *V. lecanii* (Gindin *et al.* 1996). However, *B. bassiana* grew and sporulated at temperatures of up to 30 - 32°C (Gindin *et al.* 1996). Growth of *M. anisopliae* slowed dramatically and sporulation stopped, as temperature was increased from 25°C to 30 - 32°C (Gindin *et al.* 1996). Both *B. bassiana* and *M. anisopliae* survived up to 7 days at a temperature of 36°C and 1 – 2 days at 40°C (Gindin *et al.* 1996) and growth of *V. lecanii* ceased completely at 31 - 32°C. *Beauveria bassiana and M. anisopliae* both quickly regained the ability to sporulate soon after the temperature dropped under 30°C (Gindin *et al.* 1996). *Beauveria bassiana* is thus a desirable candidate for biological control of WFT as it can persist and reproduce in extreme environments and quickly recover from the effects of such conditions.

Other research projects have explored the efficacy of commercially available or native strains of *B. bassiana* in the laboratory. Murphy *et al.* (1998) recognized the potential of commercial *B. bassiana* formulations, using Emerald

BioAgriculture's (formerly Mycotech Corporation) proprietary strain GHA, as a biocontrol agent for WFT. Fry *et al.* (1999) and Fry (2003) conducted similar studies comparing this product with native-Albertan and with non-native strains, against WFT.

I evaluated the effects of 30 strains of *B. bassiana* isolated from insects from various locations throughout Alberta, Canada, on adult female WFT, using the commercially available strain, GHA, as an internal standard, for the purpose of producing marketable strains for commercial use as microbial insecticides.

2.2 Materials and Methods

2.2.1 Greenhouse Compartment

A greenhouse compartment, 6.5 x 6.5 x 10m, was used to rear plants used for bioassays and insect rearing. The compartment had a wood and metal frame with a concrete floor. Each bench was positioned 1m from the floor and drained with a catchment gutter lying directly below (Figure 2-1A). Temperature was controlled via automated vents at the top of the roof and bottom-up steam-heating. Applying whitewash to the roof plates provided shading during hot summer days. Weeds were controlled by regular removal, washing, and addition of salt and lime to cracks in the concrete floor and to catchment gutters beneath each bench. End-to-end air circulation was maintained with two Airworks® (Airworks, North Hollywood, California) 56cm box fans, on a 13:11 L:D regimen, controlled by a Noma™ (Noma Corporation, Southfield, Michigan) outdoor light timer. An Intermatic® (Intermatic, Inc. Intermatic Plaza, Spring Grove, Illinois)

automated watering system was wired and plumbed into the existing infrastructure (Figures 2-1B, C, D).

2.2.2 Bush Beans

Phaseolus vulgaris L., McKenzie Tendergreen™ improved bush bean seeds (McKenzie Seeds, Brandon, Manitoba) were sown weekly in fourteen 60 x 30cm flats topped with Pots and Plants™ (Altwin Distributors, Medicine Hat, Alberta) seedling starter mix. The flats were arranged on the bench in 2 rows of 7 columns, with 22 seeds per flat (Figure 2-2A). Beans were watered every second day and were fertilized by hand weekly, with bulk general-use 17-5-19 fertilizer, at a rate of 0.5g/L tap water. Beans were allowed to grow for 17 days at 25±5°C, 45±10%RH, 13:11 L:D. At 18 days, the beans were transferred to the laboratory at 23±2°C, 60±5%RH, 16:8 L:D (Figure 2-2B). Seedlings were allowed to grow only the first true leaves, and axillary buds were excised to maintain full nutrient availability to these leaves. At 19 and 22 days, leaves were removed and used for thrips rearing, and at 23 days, additional healthy and unblemished leaves were used for bioassays. After use, all beans were removed from the flats, and the soil autoclaved and reused. New generations of beans were started weekly.

2.2.3 Propagation of *B. bassiana*

Isolates of *B. bassiana* were obtained from three sources: 1) from forest tent caterpillars, *Malacosoma disstria* Hubner (Lepidoptera: Lasiocamipdae) collected by Dr. B. A. Keddie, Department of Biological Sciences, University of Alberta; 2) from insects collected in the field by Matthew Greif, Ph.D. candidate,

Currah Laboratory, University of Alberta; and 3) from samples donated by Dr. Kenneth Fry, propagated in laboratories of the Alberta Research Council, Vegreville, Alberta. All isolates were cultured in petri dishes on half-strength (0.5x) Sabouraud dextrose agar (1.5% final agar concentration) plus 0.5% yeast extract (0.5x SDAY) in the dark at 23±2°C, 100%RH, for 14 days (Rajput, Master's thesis in prep.). Mature cultures were allowed to dry at 23±2°C, 60±5%RH, in complete darkness for 2 days in passive drying chambers, consisting of inverted aluminum foil casserole dishes (Figure 2-3). Plates of single isolates were evenly spaced within these chambers, with lids slightly open, allowing maximum diffusion of moisture from plates while preventing aerial contamination and light penetration (Rajput, Master's thesis in prep.). Following drying, cultures were harvested into other containers by scraping plates, and either used immediately for bioassays, or placed in cold storage (Rajput, Master's thesis in prep.).

2.2.4 Thrips Rearing

Western flower thrips were reared in twenty 1.89L Gladware® (The Glad Products Company, Oakland, California) containers. Containers were modified for air circulation by cutting a 3x15cm rectangular hole in the lid, and covering the hole with 80µm-mesh-Nitex® (Sefar America Incorporated, Depew, New York) nylon fabric glued in place with clear silicone rubber sealant (Figure 2-4). Each chamber was lined with one 4 x 17cm piece of Padco® (Padco Corporation, Washington, DC, Maryland) absorbent non-sterile cotton batting, followed by two 11 x 17cm pieces, the top piece wrapped in a layer of cheesecloth providing a supportive matrix. Each chamber received a single 400mL infusion of deionized water. A pointed scoopula was used to drill a dimple through the surface of the

cotton/cheesecloth matrix 4cm from one end of the chamber, down to its floor. Large, blemish-free, 19-day old bean leaves were cut leaving each with a 3cm petiole. The petiole of one bush bean leaf was inserted into the dimple of each arena. The cotton was then packed around the petiole. Each leaf was dusted once with non-viable Antles apple pollen (Antles Pollen Supplies, Inc. Llp., Wenatchee, Washington), covering 2 x 2cm in the central area of the leaf to provide a protein source for the thrips (Figure 2-4). Once the arena was provisioned, 35-50 female adult thrips from a 21-day-old colony were added to each arena. After an arena was closed, it was placed within an upright Sanyo (Sanyo Electric Corporation Limited, Moriguchi City, Osaka) MIR cooled growth cabinet. A total of 20 chambers were used to rear each generation. New chambers were placed at the bottom of the incubator, and older ones were shuffled to the uppermost shelves; all were incubated at 25 ± 1°C, 60±5%RH, 16:8 L:D. After a 72 hour oviposition period, all adult thrips were removed from the newest chambers and terminated. Two new, large, 22-day-old, blemish-free bean leaves were placed in each of the newest chambers, one above and one below the existing bush bean leaf for emerging larvae. Larvae were allowed to mature for 18 days to the adult stage at which time they were used for bioassays; remaining adults were used for continuing the colony at 21 days.

2.2.5 Buffers

Phosphate buffers were used to store, apply and preserve conidia.

Stock buffer solutions, prepared and stored at 4°C, included: 1) 0.2M NaH_2PO_4-H_2O stock solution: 13.8g NaH_2PO_4-H_2O (molecular weight (m.w.) =

138.00g/mol)/500mL deionized water; 2) 0.2M Na_2HPO_4-$7H_2O$ stock solution: 26.8g Na_2HPO_4-$7H_2O$ (m.w. = 268.07g/mol)/500mL deionized water.

Working buffer solutions, prepared as required, included: 1) phosphate buffer (pH 7, 0.1M), a secondary stock, on which all working buffers were based: 39mL of stock 1) + 61mL of stock 2) + 100mL deionized water; 2) phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80, for mixing and/or storing viable conidial suspensions: 50mL of 0.1M phosphate buffer + 450mL deionized water + 50µL Tween-80; and 3) phosphate buffer (pH 7, 0.1M) + 6% ethanol, for preserving conidial samples: 91mL of 0.1M phosphate buffer + 9mL of 70% ethanol.

2.2.6 Propidium Iodide Solutions

A propidium-iodide solution, refrigerated in a foil-wrapped 2000µL microcentrifuge tube, was used for fluorescence-microscopic conidial viability analysis. The propidium-iodide stock solution, prepared every 6 months, contained 3mg propidium-iodide/1mL deionized water, after Firstencil *et al.* (1990).

The propidium-iodide working solutions, prepared the day of the conidial viability assessment, contained 12µL propidium-iodide stock/1mL deionized water, after Firstencil *et al.* (1990).

2.2.7 Leaf-Disc Bioassay

2.2.7.1 Spray Tower

A spray tower was fabricated out of a 65cm length and 30cm diameter clear extruded acrylic tubing, with a thickness of 5mm. An 85cm length of a 1cm diameter solid steel rod was glued to the outside of the tube, parallel to its axis to serve as an attachment site for an airbrush. An Aztek® (Testors Corporation, Rockford, Illinois) airbrush was secured to the metal rod with an epoxy-coated metal clamp, pressurized by the laboratory air source, and operated at 10 pounds per square inch (Figure 2-5A). The entire tower was capped with a 30cm diameter funnel, with the effluent end attached to a vacuum line. The vacuum line was attached to a glass tube which ran through a stopper situated in the mouth of a bleach-filled sidearm vacuum flask. A 10% bleach solution was used for the vacuum system. The arm of the sidearm vacuum flask was attached to the laboratory vacuum line. The vacuum line was turned on before a bioassay to create negative pressure in the spray tower to limit escape of airborne conidia (Figure 2-5B). A clear acrylic disc, 5mm thick, was fitted into the bottom of the tower and served as a removable holder for five, 3.5cm petri dish thrips-arenas, arranged in a circle in the center of the disc (Figure 2-5C). Before each new bioassay, the spray tower was checked by spraying water onto water-sensitive paper (Art Advantage, Taiwan) to ensure that droplets uniformly covered the target area. The entire spray tower was positioned in the center of the fume hood within which suspensions of conidia were sprayed onto insect arenas.

2.2.7.2 Preparation for Bioassay

2.2.7.2.1 Viability Assays

Thirty-five sterile, 2000µL microcentrifuge tubes filled with approximately 2000µL phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 were set aside for preparation of conidial suspensions for each bioassay. In addition, 2.5 x 15cm test tubes were filled with 10mL phosphate buffer (pH 7, 0.1M) + 6% ethanol, prepared for preservation of conidial samples sprayed on 18 x 18mm coverslips. These samples were gathered to assess the number of conidia delivered during the bioassays.

One day prior to each bioassay, samples of conidia from selected *B. bassiana* isolates were added to 2000µL microcentrifuge tubes. Phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 was added to the test tubes until an approximate concentration of $1.0x10^7$ conidia/mL was attained. Each suspension was vortexed in a darkened room, and a 7.0µL aliquot was mixed with an equal volume of propidium-iodide working solution on a pre-cleaned microscope slide, which was covered with a 22 x 22mm coverslip. Each slide was transported in a sealed dark container to a Reichert-Jung (Kandel Electronics, Incorporated, Oreland, Pennsylvania) Polyvar microscope. This microscope was set up for epifluorescence microscopy, and was used for viability analyses, based on the epifluorescence of propidium-iodide. Conidial suspensions of approximately $1.0x10^7$ conidia/mL were used to determine viability. A combination of 10x ocular magnification with 1.25x internal and 25x objective magnifications were used, and approximately 500 conidia were visible within the field of view. This allowed for easy and rapid counting via push-button counter. One thousand conidia

were counted per isolate. Conidia fluorescing red were scored as non-viable because such conidia have non-functioning cell membranes which allow penetration of propidium iodide (Firstencil *et al.* 1990). Conidia remaining completely dark were scored as viable because such conidia have functioning, selective cell membranes which reject propidium iodide (Firstencil *et al.* 1990). The percentage of viable conidia was then calculated by dividing the number of conidia not fluorescing red, by the total number of conidia counted, and then multiplying by 100. Viability of conidia assessed in this manner was corroborated by comparisons with fluorescein-diacetate fluorescence-microscopic viability analysis, whereby living cells glow green through fluorochromasia of fluorescein diacetate and are scored as viable, after Firstencil *et al.* (1990), as well as plate counts, whereby germinating conidia are scored as viable, after Fry *et al.* (1999) and Fry (2003).

2.2.7.2.2 Preparation of Conidia and Bioassay Arenas

Dried scrapings of each selected *B. bassiana* isolate were transferred with a metal spatula into 1.6 x 15cm sterile glass test tubes. Approximately 5mL phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 was added to the test tubes and the mixtures vortexed. The conidia-buffer mixtures were then filtered through funnels equipped with 200µm-mesh nylon in-line filters via agitation by metal spatula into autoclaved 1cm diameter 7mL screw-on cap biofluid vials and labelled. Final volume was adjusted with phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80, added until an approximate concentration of $\geq 1.0 \times 10^9$ conidia/mL was attained (Rajput, personal communication).

All spray arenas were prepared at the same time as preparation of conidial suspensions. All thrips arenas were composed of inverted 3.5cm BD Falcon™ (BD Biosciences Mississauga, Ontario) petri dishes, each with one 5mm diameter access hole in the center of the "top", and one 3mm diameter 80µm-mesh-Nitex®-filtered vent hole between the access hole and outer edge of the "top" (Figure 2-6). All arena "bases" received two 3.5 cm Fisherbrand® (Fisher Scientific International, Hampton, Virginia) P8 coarse, fast flow rate filter papers. Four sets of five arenas were prepared for each isolate tested. Each set within an isolate was labelled for one of four concentrations tested, $1.0x10^6$, $3.3x10^7$, $6.7x10^7$, and $1.0x10^8$ conidia/mL. Thus, each isolate tested required a total of twenty arenas. The control consisted of five arenas labelled for phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 only.

2.2.7.2.3 Preparation of Corrected Conidial Suspensions, and Food Sources for Bioassay Arenas

Control solutions consisted only of a 2000µL aliquot of the carrier solutuion, phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80.

Concentration of all uncorrected conidial suspensions was assessed with a Reichert Neubauer (Reichert Incorporated, Depew, New York) Bright-Line hemocytometer. After correction for viability, dilutions were made, which resulted in 1500µL of four suspensions at $1.0x10^6$, $3.3x10^7$, $6.7x10^7$, and $1.0x10^8$ conidia/mL for each isolate being assayed. Each suspension was mixed in autoclaved, 1cm diameter, 7mL screw-on cap biofluid vials, arranged in a test-tube rack for isolate and concentration, and labelled.

GHA (Emerald Bioagriculture, Butte, Montana), received as an unformulated dry condial sample, served as an internal standard prepared as all other isolates. Upon receipt, this sample was subcultured once on 0.5x SDAY (without antibiotics) to confirm purity.

Eight-hundred microlitres of sterile, deionized water were added to each arena filter paper. Leaf discs, 3.5cm in diameter, were excised from whole bean leaves, placed into the arena bases, and the arena tops were immediately replaced.

2.2.7.3 Initiation of Bioassay

2.2.7.3.1 Bioassay

The bioassay was conducted in a completely randomized design with 5 replicate arenas per rate, per isolate.

Female WFT from several rearing containers of the same generation were pooled into a single container. The airbrush was primed with 200µL of the control carrier solution, followed by removal of all arena lids. Thrips arena bases, with leaf-discs, were set into the spray tower base. The spray tower base was then positioned into a permanently marked space in the fume hood; the spray tower was placed upon its base and 800µL of control solution were applied to the arena bases. All thrips arenas were removed from the base, covered with their lids, and sealed shut with clean parafilm. Five female thrips, chosen randomly from the pooled group, were inserted into each arena. The same process was repeated for each of four rates of a particular isolate proceeding from lowest to highest concentration. An 18x18mm coverslip was placed at the center point of

the spray tower base containing the arenas receiving the highest concentration of conidia. After spraying, the coverslip was removed, inserted into one vial containing 10mL phospate buffer (pH 7, 0.1M) + 6% ethanol, labelled, and vortexed. The entire spray apparatus was sterilized with 70% ethanol and 10% bleach between each isolate, and airbrush parts were rinsed with sterile, deionized water after sterilization; spray intervals and times of exposure were recorded. All arenas were incubated at 25±1°C, 100% RH, 16:8 L:D for the duration of the bioassay.

2.2.7.3.2 Observations During the Bioassay

All petri dishes were checked for mortality (Figure 2-7) in the same order in which they were sprayed starting 24 hours from the bioassay, then every 24 hours for 5 days. Conidia from coverslips were counted as per preparation of conidia. To ensure *B. bassiana* was indeed killing thrips, cadavers were surface sterilized with 2 drops 0.25% bleach, 2 drops 70% ethanol, 2 drops deionized water, and incubated on selective media (Figure 2-8).

2.2.8 Statistical Analysis

Mortality data from the leaf-disc bioassays were subjected to Probit Analysis (for median lethal concentrations, LC_{50}'s, and median lethal thresholds, LT_{50}'s) and Analysis of Variance (ANOVA) using SAS (SAS Institute, Cary, North Carolina) PROBIT and GLM, respectively. The primary purpose of subjecting the data to ANOVA was to compare the effects of the internal standard, GHA, among and within bioassays. Three types of ANOVA were run: 1) comparison of

internal standard, GHA, among all trials; 2) comparison of all isolates within pooled trials, if the effects of the internal standard were not significantly different among trials; and 3) comparison of each isolate and internal standard within each trial.

2.3 Results

2.3.1 Screening Isolates of *B. bassiana* for Efficacy Against WFT

2.3.1.1 Probit Analysis and Analysis of Variance

All isolates used were tested against a negative control group (carrier only), as well as an internal standard, GHA. A total of 15 screening trials were performed, and the results were subjected to Probit Analysis and Analysis of Variance. All trials analyzed had control mortalities of $\leq$10%, with the exception of one, with a control mortality of 12%.

2.3.1.2 Results of the Screening Trials

Selected trials were compared on the basis of similarity of cumulative mortalities of WFT caused by GHA (internal standard) across trials via Probit Analysis and ANOVA (SAS Institute Cary, North Carolina). There were no significant differences among performance of GHA across trials 2, 3, 4, 5, 6 and 7, or trials 1, 2, 3, 5 and 6. Two distinct groups were prevalent (Tables 2-2 and 2-3). Isolates were also compared within each trial separately (Table 2-4).

Two groups of isolates, one including isolates which appeared to be more virulent than, and one including those isolates which appeared equally virulent to or less virulent than GHA, were apparent from the results. Isolates 36, 2, 24, 41, 22 and 19 had LC_{50}'s in the order of 10^6, thus as a group, these isolates required the lowest dose to kill 50% of thrips. Isolates 7, 11, 15, 35, 9, 12, 13, 16, 21, 25, 23, 1, 3, and GHA had LC_{50}'s in the order of 10^7, thus as a group, these isolates required a higher dose to kill 50% of thrips. Isolates 36, 15, 2, 24, 35, 41, 13, 21, 22, 25, 19, 23, 1, and 3 had LT_{50}'s less than 5 days, thus as a group, these isolates required the least amount of time to kill 50% of thrips. Isolates 7, 11, 9, 12, 16, and GHA had LT_{50}'s greater than 5 days, thus as a group, these isolates required a greater amount of time to kill thrips. Isolates with at least one higher-virulence and at most one lower-virulence Probit-derived index (e.g. an isolate which had an LC_{50} in the order of 10^7 but had an LT_{50} less than 5 days) were deemed as more virulent than GHA. Thus isolates 36, 15, 2, 24, 35, 41, 13, 21, 22, 25, 19, 23, 1, and 3 were designated as possibly more virulent than GHA. Isolates 7, 11, 9, 12 and 16 were designated as possibly equally virulent to or less virulent than GHA (Table 2-1). All isolates were further characterized on the basis of growth morphology and mycotoxin production (Rajput, Master's thesis in prep.).

The cumulative mortalities of WFT caused by isolates deemed as possibly more virulent than isolate GHA were significantly different from the cumulative mortalities caused by GHA, with the exception of isolates 13, 3, and 1(Table 2-4). The cumulative mortalities of WFT caused by isolates deemed as possibly equally virulent to or less virulent than isolate GHA were not significantly different from those caused by GHA (Table 2-4). However, all isolates were further characterized on the basis of growth morphology and mycotoxin production (Rajput, Master's thesis in prep.).

Differences between rates used were not significant for pooled GHA comparisons across trials (Table 2-2), and significant only within trials 2, 3, 5 and 7 (Table 2-4). Differences between rates were not significant within trials 4 and 6, and especially so within trial 1.

Within these trials, isolate GHA performed poorly, resulting in extremely weak or non-discernable dose responses (N.D.R.) which varied among trials (Table 2-1); these extreme results for rate of GHA affected the overall results for rate, and in addition this was an indicator of the inconsistent effect that this isolate may have on WFT (Table 2-4).

2.3.1.3 Selection of More Virulent Isolates from Screening Trials

A total of 4 Alberta *B. bassiana* isolates, including isolates 15, 35, 19, and 3, deemed as possibly more virulent than GHA were selected, according to virulence exhibited in screening trials and conidia production (Rajput, personal communication; Tables 2-1 and 2-4). In addition, isolate 37 was included, according to growth characteristics, conidia production, and performance in a separate study (Rajput, personal communication; Fry, 2003).

2.3.2 Evaluation of Selected Strains

2.3.2.1 Group 1 of Virulence Evaluation

Isolates 15, 35, 19, and 3, selected from the screening trials, were again tested against GHA. The experiment was replicated 3 times from production of conidia, insects, and plants, to assessment of viability, all steps of spore

preparation, and bioassay. All trials analyzed had control mortalities of ≤10%, with one exception, which had a control mortality of 12.6%.

2.3.2.1.1 Performance of Isolates in Group 1

Evaluation trials were compared on the basis of similarity of cumulative mortalities of WFT caused by GHA (internal standard) across trials via Probit Analysis and Analysis of Variance. There were no significant differences among cumulative mortalities of WFT caused by GHA across evaluation trials 1a, b, and c (Table 2-6). Trials not significantly different via performance of GHA, were pooled, and all isolates within the pooled data were compared to pooled GHA (Table 2-7).

Isolates 3 and 35 performed well in comparison to GHA among the top 3 isolates out of 6 for all replicate evaluation trials and pooled data. Isolate 37 performed moderately well in comparison to GHA for all replicate evaluation trials and pooled data. Performance of isolate 15 was consistent with or poorer than that of GHA for all replicate evaluation trials and pooled data, which was contradictory to that observed by the screening trials. This isolate, though deemed as possibly more virulent than GHA, was more difficult to produce than the other isolates deemed as possibly more virulent than GHA. Performance of isolate 19 was highly variable, averaging between that of isolates 35 and 37 for the pooled data (Tables 2-5 and 2-7); performance of GHA was very consistent for replicate evaluation trials 1a, b, c.

Differences between rates used were not significant for GHA comparisons across replicate evaluation trials (Table 2-6). The difference between rates across pooled replicate evaluation trials was highly significant (Table 2-7).

One additional Alberta *B. bassiana* isolate deemed as possibly more virulent than GHA, and 2 additional Alberta *B. bassiana* isolates deemed as possibly less virulent than GHA were selected according to virulence, and ease of production, including isolates 25, 7 and 11 respectively (Rajput, personal communication; Tables 2-5 and 2-7).

2.3.2.2 Group 2 of Virulence Evaluation

The isolates 25, 7 and 11 selected from screening trials were tested against GHA as per evaluation trials 1a, b, c. All trials analyzed had control mortalities of ≤10%.

2.3.2.2.1 Performance of Isolates in Group 2

Evaluation trials were compared on the basis of similarity of cumulative mortalities of WFT caused by GHA (internal standard) as per replicate evaluation trials 1a, b, c. There were no significant differences among cumulative mortalities of WFT caused by GHA across evaluation trials 2a, b, c (Table 2-9). Trials, which were not significantly different via performance of GHA, were pooled, and all isolates within the pooled data were compared to pooled GHA (Table 2-10).

Isolate 25 performed well in comparison to GHA for all replicate evaluation trials and pooled data. Performance of isolate 7 was better in comparison to GHA for all replicate evaluation trials and pooled data, which was contradictory to that observed by the screening trials. The performance of isolate 11 was consistent with or poorer than that of GHA for all replicate evaluation trials and pooled data (Tables 2-8 and 2-10).

Differences between rates used were not significant for GHA comparisons across replicate evaluation trials (Table 2-9). The difference between rates across pooled replicate evaluation trials was highly significant (Table 2-10).

2.4 Discussion

These results extend the work of Fry *et al.* (1999) and Fry (2003) by screening several strains of *B. bassiana* for efficacy against WFT. Some isolates previously tested by Fry *et al.* (1999) were also evaluated in this project. This provided an opportunity to compare results attained by slightly different experimental procedures and performed in two separate laboratories.

2.4.1 *Beauveria bassiana* Screening and Evaluation of Results

Isolates of *B. bassiana* must be screened in an effective manner to determine which isolates perform better than, as well as, or poorer than the widely available commercial products from Emerald BioAgriculture, based on GHA.

Only conidial samples with the highest possible viabilities were used, with none below 45% viability. If conidia with viability lower than 45% were used, the corrected concentration of viable conidia would clog the spray system (Tables 2-11 and 2-12). Trials with control mortalities less than or equal to 13% were retained for further analysis (Tables 2-11 and 2-12). To ensure the fungus was indeed killing thrips, trials with control mortalities of 13% or less, and those whose cadavers produced *B. bassiana* after surface sterilization and incubation on selective media, were retained for analysis (Figure 2-8). Also, to ensure that consistent

numbers of conidia were being delivered through the airbrush for each isolate of each bioassay, conidia washed from the 18x18mm coverslips were counted (Tables 2-13 and 2-14). These "coverslip counts" only provide information on the consistency of conidia deposited at the highest rate among trials, and cannot be used to determine the dosage reaching the leaf-discs. There were no significant differences among the amounts of viable conidia reaching the leaf-disc surface at the highest rate sprayed (Tables 2-13 and 2-14). Though statistically insignificant, differences of up to 20-fold were observed among coverslip counts. Position of the airbrush and its spray settings were checked before each bioassay with water-sensitive paper; however, the dynamics of droplet deposition may have varied slightly among sprays. Though the volume of conidial suspensions deposited may have varied slightly among concentrations and among each isolate sprayed, each WFT was exposed to the same concentration of conidia on the leaf-disc surfaces. There was slight negative correlation between concentrations of GHA conidia (corrected for viability) reconstituted from coverslips within all screening trials, and percentage cumulative mortalities of WFT caused by GHA within all screening trials (Figure 2-9). This slight correlation was reduced nearly to zero when the outlier (Trial 5) was removed; this indicates independence between mortality and conidial deposition, and because all bioassay procedures were rigouously standardized, such independence can be attributed to all other trials. To keep the experiment as objective as possible, isolate identities were provided by Sunil Rajput, propagator and custodian of the isolates, only after this research was completed (Table 2-15).

Despite rigorous quality control and standardization, results from the screening trials, forming the basis for selecting isolates for further triplicate evaluation trials, and results of the evaluation trials were not consistent for all

isolates. Of isolates 3, 15, 19, 25 and 35, which were selected on the basis of performance superior to that of GHA during the screening trials, only 3, 25 and 35 remained superior to GHA while isolate 15 was less effective in the evaluation trials. The performance of isolate 19 varied widely for each of the evaluation trials. Of isolates 7 and 11 selected on the basis of performance similar to or less effective than that of GHA during the screening trials, only isolate 11 remained less effective than GHA. Isolate 7, selected because it was less effective initially, performed better than GHA in the evaluation trials. Consistent results were only observed in 4 of the 7 isolates tested. This suggests that the number of screening trials must be increased before selecting isolates for further study. Here, the combination of screening and evaluation trials did identify three islolates more virulent to WFT than GHA, the isolate now formulated in a commercial product. One additional isolate, 37, performed moderately well in comparison to GHA, but was not chosen on the basis of screening trials.

The LC_{50}'s for isolates tested in this project, and originating from the Alberta Research Council (ARC), were compared to the LC_{50}'s obtained in a similar study by Fry (2003) (Table 2-16). The LC_{50}'s of isolates tested in this project were obtained from the average of 3 replicate evaluation trials. Isolate 35 performed similarly in this project and in the ARC project. Isolate 37 was nearly two orders of magnitude less effective in this project than in the ARC project. The exact cause for this discrepancy may include the influence of abiotic and biotic factors which differ among laboratories. This project provided further information for these two isolates by repeating the test in triplicate, allowing for a glimpse into inter- and intra-isolate variability which may affect results of this and similar studies.

Differences in performance among *B. bassiana* isolates, inter-isolate variability, may stem from the type of insect and the location from which they were isolated. This fungus is cosmopolitan, parasitizes insects, and persists in soil. *Beauveria bassiana* is also mesophilic, with an optimum temperature ranging between 20° and 30° C, and is capable of growth between 6° and 35° C; for the majority of isolates 25° C is the optimum temperature for growth (Fargues *et al.*, 1997). Differences between isolates were apparent outside the optimal temperature range, i.e. <20° C and >30° C (Fargues *et al.*, 1997). The isolates of *B. bassiana* used in this project were propagated at 23±2°C, and the bioassay arenas were incubated at 25±1°C, well within the optimal range. The wide range of thermal tolerance distinguishes *B. bassiana* from other entomopathogenic fungi and allows a researcher to select specific isolates for specific pest management requirements. However, there is no clear relationship between upper and lower temperature thresholds and geoclimatic origin (Fargues *et al.*, 1997).

In addition, differences in performance among *B. bassiana* isolates may also stem from genetic and metabolic variation. *Beauveria bassiana* has a significant degree of strain variability in production of cuticle-degrading enzymes and in the pattern of enzyme expression on different growth media (Gupta *et al.*, 1992). Variability by strain, in cuticle-degrading enzyme expression and activity has an effect on virulence and efficacy of various strains of *B. bassiana*; these factors are the most important, as they affect whether *B. bassiana* may infiltrate the exoskeleton to start the infection process. Onset and rate of infection, and host specificity vary independently among strains (Gupta *et al.*, 1994). Other virulence factors include effects of toxic metabolites such as beauvericin. By strain selection or genetic modification, *B. bassiana* may be improved as a

biocontrol agent by selecting appropriate strains for appropriate pest groups (Gupta *et al.*, 1992 and 1994).

Differences in performance within *B. bassiana* isolates, intra-isolate variability, may be caused by random mutation. Such variability may also stem from heterokaryosis, a process occurring readily in deuteromycetes, ascomycetes, and basidiomycetes (Carlile and Watkinson, 1995). Hyphae from the same colony or same species may fuse by anastomosis. If the two colonies are genetically dissimilar, a heterokaryon may result (Carlile and Watkinson, 1995). A heterokaryon differs from both parent colonies with respect to growth and other metabolic characteristics, since it will have genetic information from both homokaryons (Carlile and Watkinson, 1995). In rare instances, two haploid nuclei may fuse to form a somatic diploid nucleus. During division of these nuclei, mitotic crossing over may occur. Errors may occur during crossing over, resulting in 2n-1 and 2n+1 aneuploid daughter nuclei, which commonly cause poor growth. Metabolic processes are negatively influenced within fungal cells where daughter nuclei have either lost or gained chromosomes (Carlile and Watkinson, 1995). Uninucleate spores containing a recombinant haploid nucleus produce a recombinant homokaryon which differs from both original homokaryons (Carlile and Watkinson, 1995). The process resulting in such recombinant strains is referred to as the parasexual cycle (Carlile and Watkinson, 1995). The parasexual cycle has been observed in *B. bassiana*, and described by Paccola-Meirelles and Azevedo (1991).

It is possible that some *B. bassiana* isolates tested in this project may have undergone some form of heterokaryosis and possibly parasexual recombination, resulting in the differential results observed for some isolates between replicate trials. *Beauveria bassiana* may well be a complex of species or genotypes,

further complicating these trials (Fargues *et al.*, 1997). Genetic and metabolic analyses should therefore be included to confirm the identity of an isolate and its purity before use in bioassays. This will help produce consistent results and increase their accuracy and precision.

Means of conidial delivery are of paramount importance as the quality of coverage will ultimately affect how many conidia will contact a host. Samples of the number of conidia received from the highest rate sprayed were obtained from 18x18mm coverslips. Both the screening and evaluation trials were treated with 800µL of $1.0x10^8$ conidia/mL. The airbrush and nozzle were flushed in two directions during cleansing to ensure the system never clogged. The concentration of conidia recovered from coverslips was consistent between trials; however loss of conidia must be taken into account when developing spraying techniques, accessories, formulations, and especially rates.

2.5 Summary

Isolates 3, 35, 7, and 25 were more virulent than GHA in 3 replicate evaluation trials, whereas isolate 37 was moderately more virulent than GHA, and isolates 15, 19, and 11 less virulent than or similarly virulent to GHA. These and other isolates must be screened rigourously, i.e. replicated at least three times in a highly standardized fashion. Replication of screening assays will confirm the status of the isolates, and help to uncover hidden variation within isolates. Both inter- and intra-isolate variability affect the performance of an isolate in time and space. The difference in performance among and within isolates, tested both in this project and at the ARC, show that standardization is a requirement for different laboratories screening isolates of *B. bassiana*, to reliably compare results

obtained from different laboratories. The isolates performing better than GHA in this study have the potential to be developed into microbial control agents for WFT. These isolates should be further studied through more rigorous experimentation, including genetic and metabolic analyses, which will help confirm their identity and their purity before use in bioassays.

2.6 Tables and Figures

Table 2-1. Performance of *B. bassiana* isolates tested on WFT, among screening trials (N.D.R. = non-discernable dose response, LC_{50} = conidia/mL, LT_{50} = days).

Trial	Isolate	LC_{50}	LT_{50}
1	7	4.38E+10	5.56
	36	6.36E+06	4.35
	GHA	N.D.R.	7.42
2	11	5.96E+07	5.32
	15	1.30E+07	4.78
	GHA	4.92E+09	5.72
3	2	2.40E+06	4.25
	24	4.86E+06	4.57
	35	3.06E+07	4.95
	41	1.22E+06	4.52
	GHA	N.D.R.	7.08
4	9	8.23E+07	5.28
	12	6.25E+07	5.31
	13	3.00E+07	4.86
	16	9.05E+07	5.39
	GHA	N.D.R.	5.91
5	21	1.24E+07	4.56
	22	1.49E+06	3.47
	25	1.04E+07	3.88
	GHA	1.52E+39	6.63
6	19	5.78E+06	4.39
	23	1.48E+07	4.43
	GHA	1.05E+18	5.34
7	1	1.59E+07	4.66
	3	1.11E+07	4.50
	GHA	8.51E+07	5.11

Table 2-2. Variability of cumulative mortality (%) of WFT caused by isolate GHA (internal standard) among screening trials (Tukey Means Test, $\alpha = 0.05$, N = 4); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Trial
36.15 a	5.79	4
34.25 a	5.79	7
29.71 a b	5.79	2
21.51 a b	5.79	3
16.03 a b	5.79	5
10.20 a b	5.79	6
5.68 b	5.79	1

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Trial	6	4.23	0.0079
Rate	3	1.82	0.1796

Table 2-3. List of screening trials and isolates comparable on the basis of performance of the internal standard, GHA.

Comparable Trials		Isolates Within Trial
1	Group 1	7, 36
2		11, 15
3		2, 24, 35, 41
5		21, 22, 25
6		19, 23
2	Group 2	11, 15
3		2, 24, 35, 41
4		9, 12, 13, 16
5		21, 22, 25
6		19, 23
7		1, 3

Table 2-4. Comparison of cumulative mortality (%) of WFT caused by *B. bassiana* isolates within each screening trial (Tukey Means Test, $\alpha = 0.05$, N = 4); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	$\pm SE_{Trial}$	Trial	$\pm SE_{Isolate}$	Isolate
56.53 a	6.94	1	5.70	36
32.44 a b	6.94		4.80	7
5.68 b	6.94		4.00	GHA
56.88 a	5.53	2	6.50	15
39.75 a b	5.53		6.10	11
29.71 b	5.53		5.70	GHA
66.98 a	8.83	3	6.40	24
66.61 a	8.83		5.20	2
60.26 a b	8.83		8.10	41
50.68 a b	8.83		8.50	35
21.51 b	8.83		3.60	GHA
54.58 a	10.98	4	8.50	13
41.56 a	10.98		6.10	12
41.25 a	10.98		6.80	16
40.68 a	10.98		6.20	9
36.15 a	10.98		5.70	GHA
72.12 a	9.81	5	5.60	22
64.73 a	9.81		8.50	25
59.24 a b	9.81		7.70	21
16.03 b	9.81		3.70	GHA
70.91 a	10.81	6	7.50	19
60.87 a	10.81		8.60	23
10.20 b	10.81		3.80	GHA
62.70 a	6.71	7	7.60	3
60.40 a	6.71		7.70	1
34.25 a	6.71		5.70	GHA

ANOVA Table (Type III S.S.)

Trial	Source	DF	F-Value	P-Value
1	Isolate	2	13.45	0.0061
	Rate	3	0.92	0.4879
2	Isolate	2	6.18	0.0349
	Rate	3	7.49	0.0188
3	Isolate	4	4.59	0.0177
	Rate	3	8.27	0.0300
4	Isolate	4	0.40	0.8069
	Rate	3	3.37	0.0549
5	Isolate	3	6.61	0.0118
	Rate	3	5.87	0.0167
6	Isolate	2	9.06	0.0154
	Rate	3	3.71	0.0807
7	Isolate	3	4.17	0.0415
	Rate	3	15.98	0.0006

Table 2-5. Performance of *B. bassiana* isolates tested on WFT, among evaluation trials 1a – 1c (LC_{50} = conidia/mL, LT_{50} = days).

Evaluaton Trial	Isolate	LC_{50}	LT_{50}
1a	3	2.75E+07	4.66
	15	2.33E+10	10.38
	19	2.68E+11	5.59
	35	1.86E+07	4.41
	37	3.42E+07	5.08
	GHA	4.70E+08	6.43
1b	3	2.63E+07	4.95
	15	1.47E+08	6.30
	19	3.13E+07	4.68
	35	2.85E+07	4.95
	37	1.43E+08	5.87
	GHA	1.33E+09	5.24
1c	3	3.18E+07	4.58
	15	9.13E+07	6.46
	19	2.19E+07	4.92
	35	3.31E+07	5.04
	37	1.02E+08	5.66
	GHA	3.96E+09	6.42

Table 2-6. Variability of cumulative mortality (%) of WFT caused by isolate GHA (internal standard) among evaluation trials 1a – 1c (Tukey Means Test, $\alpha = 0.05$, N = 4); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Evaluation Trial
19.08 a	3.78	1c
18.20 a	3.78	1b
17.16 a	3.78	1a

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Evaluation Trial	2	0.06	0.9385
Rate	3	3.85	0.0752

Table 2-7. Comparison of cumulative mortality (%) of WFT caused by *B. bassiana* isolates within pooled evaluation trials (Tukey Means Test, $\alpha = 0.05$, N = 12); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Isolate
53.57 a	5.66	3
53.26 a	5.66	35
37.02 a b	5.66	19
36.51 a b	5.66	37
18.15 b	5.66	GHA
15.38 b	5.66	15

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Isolate	5	8.43	<0.0001
Rate	3	23.43	<0.0001

Table 2-8. Performance of *B. bassiana* isolates tested on WFT, among evaluation trials 2a – 2c (LC_{50} = conidia/mL, LT_{50} = days).

Evaluation Trial	Isolate	LC_{50}	LT_{50}
2a	7	1.81E+07	4.41
	11	2.44E+10	8.09
	25	1.49E+07	4.48
	GHA	1.26E+08	6.39
2b	7	4.40E+07	4.80
	11	1.88E+08	5.48
	25	3.72E+07	4.72
	GHA	6.37E+08	5.81
2c	7	4.58E+07	5.52
	11	2.37E+08	6.33E+13
	25	5.34E+07	5.61
	GHA	8.38E+09	5.45

Table 2-9. Variability of cumulative mortality (%) of WFT caused by isolate GHA (internal standard) among evaluation trials 2a – 2c (Tukey Means Test, $\alpha = 0.05$, N = 4); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Evaluation Trial
21.15 a	5.97	2b
21.10 a	5.97	2a
4.97 a	5.97	2c

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Evaluation Trial	2	2.44	0.1675
Rate	3	1.85	0.2394

Table 2-10. Comparison of cumulative mortality (%) of WFT caused by *B. bassiana* isolates within pooled evaluation trials (Tukey Means Test, $\alpha = 0.05$, N = 12); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Isolate
49.72 a	4.41	25
47.97 a	4.41	7
15.74 b	4.41	GHA
10.25 b	4.41	11

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Isolate	3	22.30	<0.0001
Rate	3	18.15	<0.0001

Table 2-11. Viability of *B. bassiana* conidia used, and WFT control mortality recorded among screening trials.

Trial	Isolate	Viablity (= % Live Conidia)
1	7	45.3
1	36	99.1
1	GHA	84.8
2	11	99.2
2	15	96.4
2	GHA	84.6
3	2	96.9
3	24	98.9
3	35	99.3
3	41	97.4
3	GHA	89.6
4	9	99.7
4	12	97.0
4	13	85.0
4	16	99.5
4	GHA	94.0
5	21	97.8
5	22	99.3
5	25	96.5
5	GHA	98.0
6	19	98.4
6	23	99.8
6	GHA	96.5
7	1	99.5
7	3	99.7
7	S-GHA	94.4
7	GHA	94.4

Trial	Percentage Control Mortality
1	12.0
2	3.4
3	4.0
4	4.0
5	8.0
6	3.4
7	0.0

Table 2-12. Viability of *B. bassiana* conidia used, and WFT control mortality recorded among evaluation trials.

Evaluation Trial	Isolate	Viablity (= % Live Conidia)
1a	3	99.9
1a	15	99.0
1a	19	89.8
1a	35	99.3
1a	37	99.3
1a	GHA	95.6
1b	3	99.8
1b	15	99.0
1b	19	99.4
1b	35	100.0
1b	37	99.5
1b	GHA	97.8
1c	3	98.8
1c	15	97.5
1c	19	98.5
1c	35	99.0
1c	37	94.7
1c	GHA	93.2
2a	7	52.1
2a	11	95.0
2a	25	88.9
2a	GHA	97.7
2b	7	90.1
2b	11	94.0
2b	25	98.2
2b	GHA	91.0
2c	7	99.9
2c	11	91.6
2c	25	93.6
2c	GHA	96.9

Evaluation Trial	Percentage Control Mortality
1a	12.6
1b	0.0
1c	8.0
2a	0.0
2b	0.0
2c	7.4

Table 2-13. Comparison of coverslip counts among screening trials ($\alpha = 0.05$).

Trial	Isolate	[Conidia]	Viability	[Conidia] Corrected for Viability
1	7	5.7E+05	45.3	2.6E+05
1	36	7.0E+05	99.1	7.0E+05
1	GHA	1.1E+06	84.8	9.4E+05
2	11	3.4E+05	99.2	3.4E+05
2	15	3.4E+05	96.4	3.3E+05
2	GHA	3.7E+05	84.6	3.2E+05
3	2	2.1E+06	96.9	2.0E+06
3	24	6.5E+05	98.9	6.4E+05
3	35	7.5E+05	99.3	7.4E+05
3	41	2.8E+06	97.4	2.7E+06
3	GHA	2.6E+06	89.6	2.3E+06
4	9	8.5E+05	99.7	8.5E+05
4	12	8.5E+05	97	8.2E+05
4	13	4.5E+05	85	3.8E+05
4	16	7.0E+05	99.5	7.0E+05
4	GHA	1.5E+06	94	1.4E+06
5	21	1.6E+06	97.8	1.5E+06
5	22	1.9E+06	99.3	1.9E+06
5	25	5.0E+05	96.5	4.8E+05
5	GHA	6.8E+06	98	6.7E+06
6	19	9.5E+05	98.4	9.3E+05
6	23	1.2E+06	99.8	1.2E+06
6	GHA	1.3E+06	96.5	1.2E+06
7	1	7.5E+05	99.5	7.5E+05
7	3	6.5E+05	99.7	6.5E+05
7	GHA	9.5E+05	94.4	9.0E+05

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Trial	6	1.63	0.1920

Table 2-14. Comparison of coverslip counts among evaluation trials ($\alpha = 0.05$).

Evaluation Trial	Isolate	[Conidia]	Viability	[Conidia] Corrected for Viability
1a	3	6.5E+05	99.9	6.5E+05
1a	15	1.0E+06	99.0	9.9E+05
1a	19	1.9E+06	89.8	1.7E+06
1a	35	9.5E+05	99.3	9.4E+05
1a	37	6.5E+05	99.3	6.5E+05
1a	GHA	1.8E+06	95.6	1.7E+06
1b	3	5.0E+05	99.8	5.0E+05
1b	15	9.5E+05	99.0	9.4E+05
1b	19	8.0E+05	99.4	8.0E+05
1b	35	1.1E+06	100.0	1.1E+06
1b	37	9.5E+05	99.5	9.5E+05
1b	GHA	8.5E+05	97.8	8.3E+05
1c	3	1.3E+06	98.8	1.2E+06
1c	15	2.1E+06	97.5	2.0E+06
1c	19	8.0E+05	98.5	7.9E+05
1c	35	9.5E+05	99.0	9.4E+05
1c	37	1.0E+06	94.7	9.5E+05
1c	GHA	1.6E+06	93.2	1.4E+06
2a	7	1.1E+06	52.1	5.5E+05
2a	11	1.1E+06	95.0	1.0E+06
2a	25	2.0E+06	88.9	1.8E+06
2a	GHA	1.4E+06	97.7	1.4E+06
2b	7	1.2E+06	90.1	1.0E+06
2b	11	1.1E+06	94.0	9.9E+05
2b	25	1.2E+06	98.2	1.1E+06
2b	GHA	1.5E+06	91.0	1.4E+06
2c	7	1.2E+06	99.9	1.1E+06
2c	11	1.4E+06	91.6	1.2E+06
2c	25	1.1E+06	93.6	9.8E+05
2c	GHA	1.5E+06	96.9	1.4E+06

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Evaluation Trial	5	0.75	0.5940

Table 2-15. Origin of *B. bassiana* isolates used in this study.

Isolate #	Owner	Insect Host (Order)
1	Keddie, University of Alberta.	Lepidoptera
2	Rajput/Keddie, University of Alberta.	Lepidoptera
3	Keddie, University of Alberta.	Lepidoptera
7	Greif, Currah Laboratory, University of Alberta.	Hemiptera
9	Greif, Currah Laboratory, University of Alberta.	Hymenoptera
11	Greif, Currah Laboratory, University of Alberta.	Hymenoptera
12	Greif, Currah Laboratory, University of Alberta.	Coleoptera
13	Greif, Currah Laboratory, University of Alberta.	Coleoptera
15	Greif, Currah Laboratory, University of Alberta.	Araneae
16	Greif, Currah Laboratory, University of Alberta.	Diptera
19	Greif, Currah Laboratory, University of Alberta.	Araneae
21	Greif, Currah Laboratory, University of Alberta.	Araneae
22	Greif, Currah Laboratory, University of Alberta.	Araneae
23	Greif, Currah Laboratory, University of Alberta.	Diptera
24	Greif, Currah Laboratory, University of Alberta.	Araneae
25	Greif, Currah Laboratory, University of Alberta.	Acari
35	Alberta Research Council, Vegreville, Alberta.	Homoptera
36	Alberta Research Council, Vegreville, Alberta.	Homoptera
37	Alberta Research Council, Vegreville, Alberta.	Homoptera
41	Greif, Currah Laboratory, University of Alberta.	Coleoptera

Table 2-16. Comparison of performance of two Alberta *B. bassiana* isolates tested rigourously in this thesis with the same tested by the Alberta Research Council (ARC).

Isolate Names		LD_{50}	
Thesis	ARC	Thesis	ARC
35	67	2.67E+07	1.37E+07
37	66	9.30E+07	2.47E+06

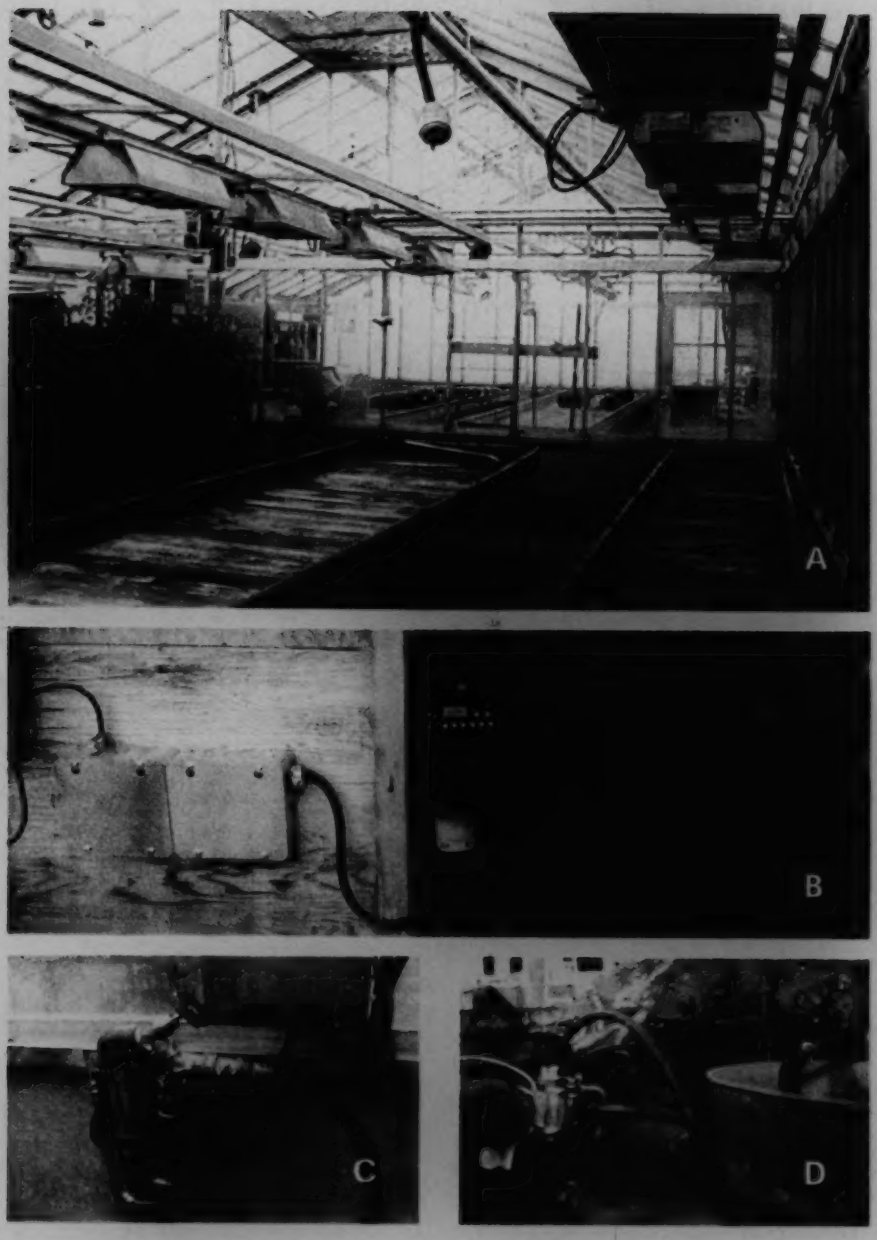

Figure 2-1. Greenhouse before bioassay plant production (A) and detail of automated watering system: watering timer and solenoid valve control (B), solenoid valve intersecting water supply (C), and drip irrigation spikes, fed by main water line and inserted into soil (D).

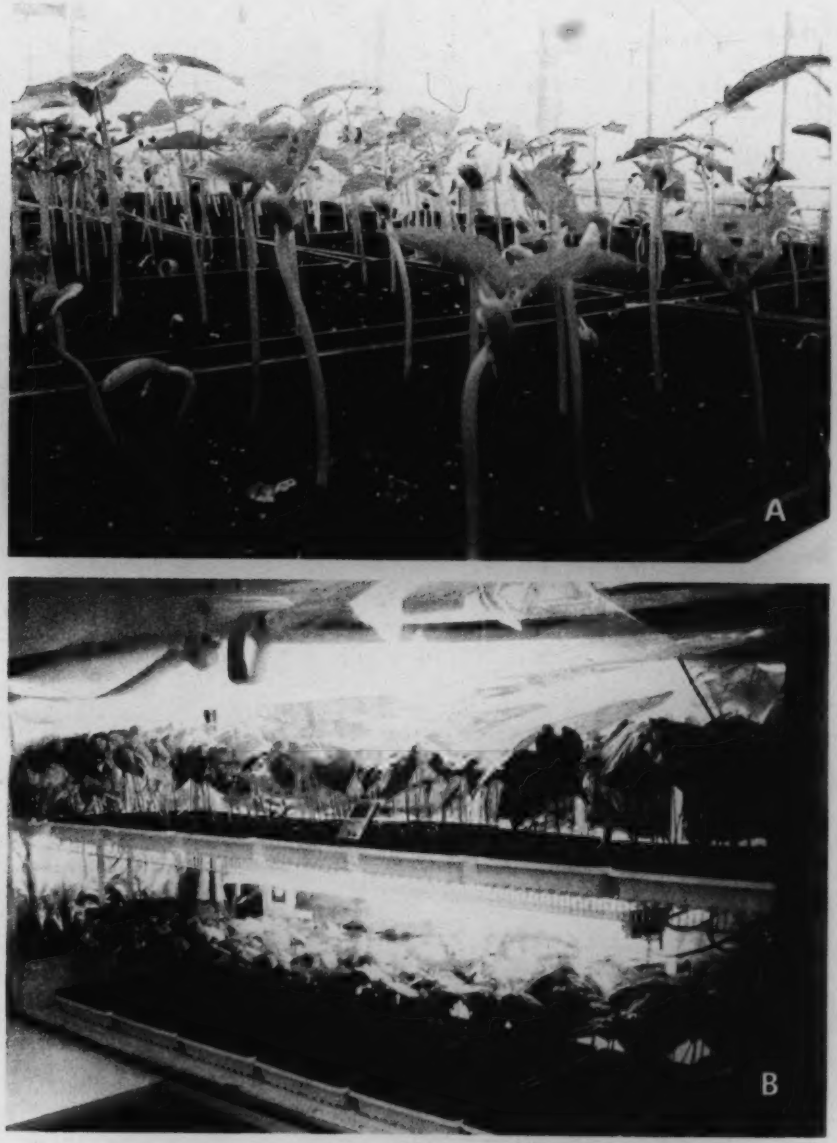

Figure 2-2. Greenhouse bush bean production (A) and laboratory bush bean environmental chamber (B).

Figure 2-3. *Beauveria bassiana* conidia production system.

Figure 2-4. Complete thrips rearing chamber (A) and detail of rearing chamber setup (B).

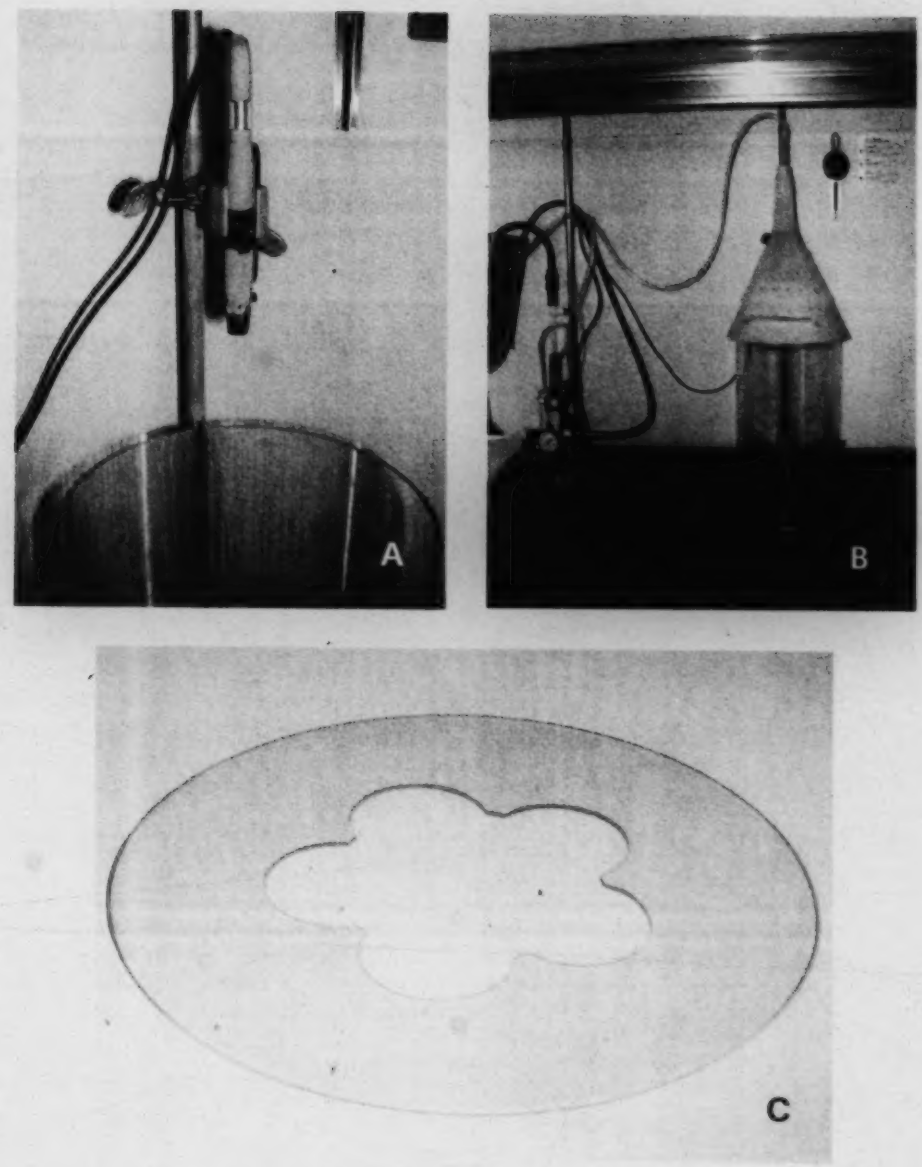

Figure 2-5. Airbrush for spraying conidial suspensions (A), complete spray chamber (B) and spray chamber base with positions for 5 arenas (C).

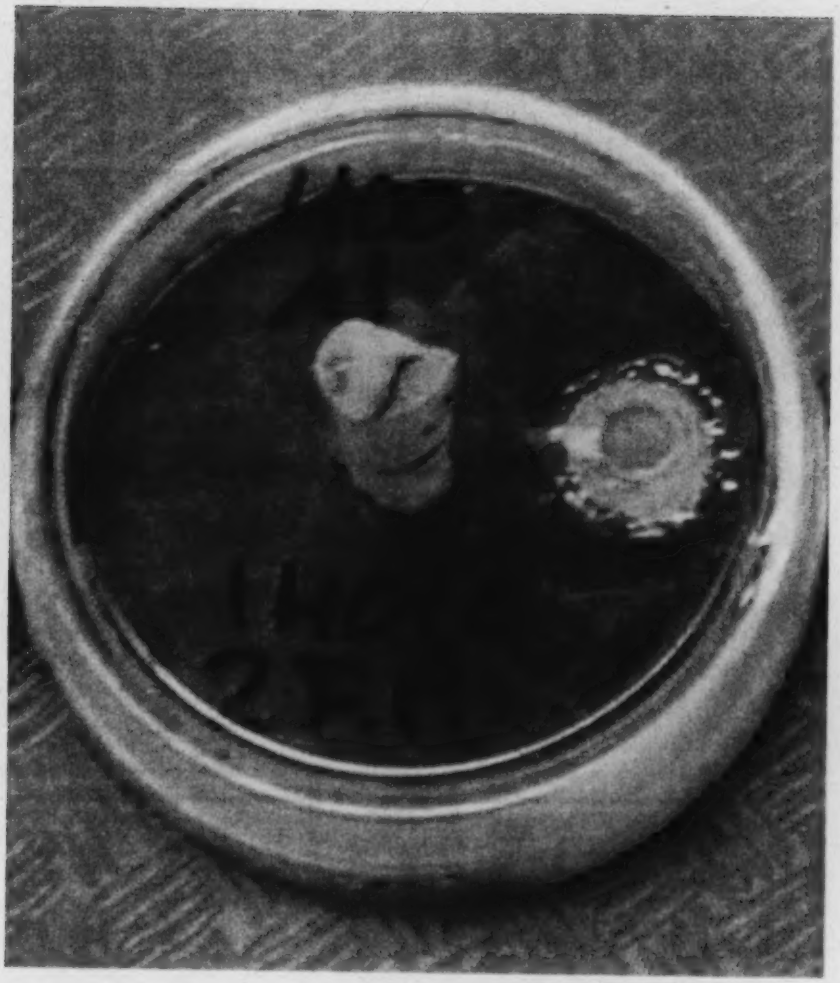

Figure 2-6. Complete bioassay arena with cork stopper and vent, containing leaf disc and WFT females.

Figure 2-7. Cadaver of WFT female killed by *B. bassiana*: hyphae re-emerged (RH ≥ 70%) and conidiophores were produced.

Figure 2-8. Cadaver of WFT female placed on media selective for *B. bassiana* only, confirming *B. bassiana* as cause of death.

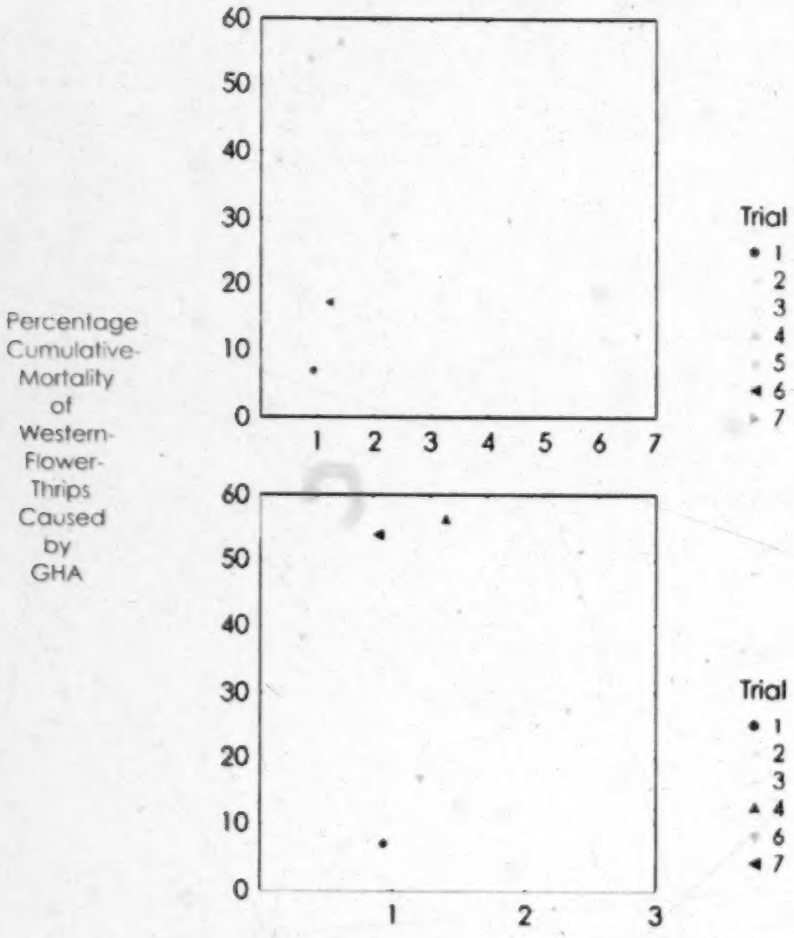

Figure 2-9. Correlation between concentrations of GHA conidia (corrected for viability) reconstituted from coverslips within all screening trials, and percentage cumulative mortalities of WFT caused by GHA within all screening trials, with outlier included (top), Pearson Correlation Coefficient = -0.407, with outlier excluded (bottom), Pearson Correlation Coefficient = -0.087.

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Chapter 3. Effects of *Beauveria bassiana* (Balsamo) Vuillemin on Cabbage Looper, *Trichoplusia ni* (Hübner), and its Natural Predator and Biological control Agent, the Predaceous Spined Soldier Bug, *Podisus maculiventris* (Say).

3.1 Introduction

Research involving biological control of cabbage looper, *Trichoplusia ni* Hübner, has been occurring for several years. Microbial biological control agents commonly used for controlling outbreaks of cabbage looper include viruses and bacteria, for which there is abundant literature on pathological effects and modes of action; less emphasis has been placed on entomopathogenic fungi (Himeno *et al.* 1985; Hostetter and Ignoffo, 1984; Keddie *et al.*, 1989; Pietrantonio and Gill, 1992; Vail *et al.*, 1971). Bioassays evaluating the fungus *Normuraea rileyi* (Farlow) Samson as a potential fungal biological control agent for cabbage looper have produced unsatisfactory results (Hostetter and Ignoffo, 1984). Parasitoid and predaceous insects also have been used as biological control agents for cabbage looper. However, to exploit them as biological control agents, additional research into behaviour, chemical ecology, functional response, movement, and population dynamics is required to formulate models describing the relationships between these insects and their host or prey (Martin *et al.* 1984).

Viral agents are commonly used to control lepidopteran pests such as cabbage looper. Nucleopolyhedrosis viruses (NPV's) have been tested on cabbage looper infesting lettuce, broccoli and cabbage (Vail *et al.*, 1971). Vail *et al.* (1971) found that a spray formulated from two diseased larvae could protect up to 1 acre of crops. Nucleopolyhedrosis viruses are suitable for

biological control of cabbage looper through their specificity and high virulence. These are double-stranded DNA viruses which have two phenotypes (Keddie and Volkman, 1985). One phenotype consists of a polyhedral envelope enclosing multiple bacilliform nucleocapsids occluded within a proteinaceous matrix; the entire structure is referred to as a polyhedron (Keddie and Volkman, 1985). This phenotype is responsible for transmitting this virus between host insects (Keddie and Volkman, 1985). The second phenotype consists of a single nucleocapsid contained within a viral-modified cellular plasma membrane formed around the virus as it buds out of an infected cell; this structure is referred to as extracellular virus (EV) or budded virus (BV) (Keddie and Volkman, 1985). This phenotype is responsible for systemic infection of the host insect (Keddie and Volkman, 1985). Keddie *et al.* (1989) found that EV infects cells in the midgut, spreads to the hemocoel, followed by hemocytes and tracheal system. Subsequent spread to other tissues is facilitated by circulating EV and contact with infected hemocytes or tracheal cells (Keddie *et al.*, 1989). This biological control agent must be ingested by the host insect to have an effect.

A bacterial agent commonly used for control of lepidopteran pests is *Bacillus thuringiensis* (*B.t.*) isolates of which are relatively specific, being toxic to a limited number of insect taxa, including lepidopterans, dipterans, and coleopterans (Pietrantonio and Gill, 1992). *Bacillus thuringiensis* produces a proteinaceous, parasporal, crystalline inclusion body known as delta-endotoxin, which dissolves in the alkaline environment of the host midgut upon injestion (Himeno *et al.* 1985; Pietrantonio and Gill, 1992). Proteolytic cleavage activates delta-endotoxin, which destroys midgut epithelium (Pietrantonio and Gill, 1992). *Bacillus thuringiensis* var. *kurstaki* produces lepidopteran-toxic crystals (Himeno *et al.*, 1985). Delta-endotoxin causes swelling of columnar and goblet cells of the

midgut epithelium and later, detachment of columnar cells from the basement membrane of the midgut (Himeno *et al.*, 1985). In addition, leakage of gut content into hemolymph disrupts osmotic balance, causing paralysis and death (Pietrantonio and Gill, 1992). Like viruses, this biological control agent must be ingested by the host insect to have an effect.

A predatory hemipteran tested as a biological control agent for control of lepidopteran pests is *Podisus maculiventris* (Say), the spined soldier bug, a generalist predator which feeds on larvae of Lepidoptera and Coleoptera in agricultural environments (Whittmeyer *et al.*, 2001). This bug has voracious feeding habits and requires a suitable quantity of high-quality nutritional resources during juvenile and adult life for optimal health and reproductive development (Whittmeyer *et al.*, 2001). *Podisus maculiventris* has been observed in the laboratory and field to attack and kill all developmental stages except eggs, of 100 insect species from 7 orders (Whittmeyer *et al.*, 2001). In addition to its search and kill capabilities, *P. maculiventris* has been shown to disseminate *T. ni* single-embedded nucleopolyhedrosis virus (TnSNPV) (Biever *et al.*, 1982). The ability of this hemipteran to disseminate pathogens specific to its prey items adds to its beneficial properties as a biological control agent.

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin infects Lepidoptera, Coleoptera, Hemiptera, Diptera, Neuroptera and Hymenoptera (Donegan and Lighthart, 1989; Fargues *et al.*, 1997; Steinkraus and Tugwell, 1997). Despite its beneficial properties as a biological control agent, this fungus can have detrimental effects on integrated pest management systems, such as those using predaceous hemipterans to control lepidiopteran pests. *Beauveria bassiana* may affect beneficials and pests differently or similarly to differing degrees. When selecting this microbial insecticide to control pest insects

in greenhouses, one must consider its effects on natural enemies whether they occur inside or outside the greenhouse.

Knowing the role of all biocontrol agents and their trophic interactions is extremely important for implementing effective integrated pest management programs. *Beauveria bassiana* effectively killed larvae of Colorado potato beetle, *Leptinotarsa decimlineata* Say in a field trial conducted by Poprawski *et al.* (1997). Foliar application of *B. bassiana* conidia alone or in conjunction with the hemipteran predator *Perillus bioculatus* (F.) provided more substantial protection against defoliation than parallel chemical or no treatments. However it was unclear as to what extent *P. bioculatus* assisted in controlling *L. decimlineata* (Poprawski *et al.*, 1997).

When predators are used as biological control agents the greenhouse grower must provide alternate or supplementary food sources and a suitable environment to support the predators, which will in turn be conducive to the longest period of their effective use. An isolate of *B. bassiana* had detrimental effects on the predaceous chrysopid neuropteran *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) placed in a high-stress regime consisting of starvation and excessive heat (Donegan and Lighthart, 1989). Starvation adversely affected the energy reserves of this predator and altered the processes in RNA synthesis, which in turn affected cuticle synthesis and the feeding response. Efficiency of the immune system was also decreased, which led to increased succeptibility to *B. bassiana* (Donegan and Lighthart, 1989). High temperature stress did not significantly affect susceptibility of *C. carnea* to *B. bassiana* but a synergistic effect resulted for adults under the starvation regime (Donegan and Lighthart, 1989).

There are also cases of biological control where synergistic control of pests occurs. For example the aphelinid wasp, *Aphelinus asychis* (Walker) (Hymenoptera), a parasitoid of the aphid, *Diuraphis noxia* (Kurdj) (Hemiptera: Aphididae), probes potential hosts with its ovipositor before ovipositing or feeding (Mesquita and Lacey, 2001). Both non-treated (control) aphids and those pre-treated with *Paecilomyces fumosoroseus* (Wize) Brown & Smith were probed by this wasp in order to determine host suitability. *Diuraphis noxia* showed increased levels of mummification if first parasitized and then exposed to fungus (Mesquita and Lacey, 2001). This indicated that *A. asychis* was able to escape the effects of this entomopathogenic fungus and evidence suggested a fungistatic substance secreted by the developing parasitoids was involved (Mesquita and Lacey, 2001). *Aphelinus asychis* demonstrated selective behavior during oviposition or feeding and relied on internal cues to determine host suitability (Mesquita and Lacey, 2001). Time between and order of exposures to parasitoids and fungus were important although there were cases where the hosts were simultaneously parasitized and exposed to fungus and still yielded an F1 generation (Mesquita and Lacey, 2001). Increased probing of infected aphids may encourage horizontal transmission of fungus between potential *D. noxia* hosts. Identifying synergistic interactions among biological control agents can lead to more effective biological control (Mesquita and Lacey, 2001). Such synergistic applications of biological control agents potentially could provide a greater degree of protection than any of these agents used alone. More importantly, predators or parasitoids resistant to or able to avoid infections caused by entomopathogenic fungi would be a wise investment for the greenhouse grower if both are to be used simultaneously in an integrated approach.

Here I evaluated the effects of one strain of *B. bassiana*, isolated from an insect collected in Alberta, and the commercially available strain (internal standard), GHA on 4th instar cabbage looper larvae, in conjunction with a natural predator and biological control agent of cabbage looper, *P. maculiventris*.

3.2 Materials and Methods

3.2.1 Greenhouse Compartment

A greenhouse compartment, measuring 6.5 x 6.5 x 10m, was used to rear plants, used for bioassays and insect rearing, as per chapter 2.

3.2.2 Bush Beans

Phaseolus vulgaris L., Mackenzie Tendergreen™ improved bush bean seeds (McKenzie Seeds, 30 - 9th Street, Brandon, Manitoba, Canada) were sown weekly in sixty15cm plant pots filled with Pots and Plants™ (Altwin Distributors, Medicine Hat, Alberta) seedling starter mix. Pots were arranged on the bench in 6 rows of 10 columns with 6 seeds per pot. Beans were watered and fertilized as per chapter 2. Beans were allowed to grow for 17 days as per chapter 2. At 18 days, two pots were transferred directly to 50 x 50 x 100cm whole-plant bioassay cages inside the laboratory at 23±2°C, 60±5%RH, 16:8 L:D (Figure 3-1). Seedlings were allowed to grow only the first true leaves as per chapter 2. At 23 days, all whole plants were used for bioassays. After use, all beans were removed from the pots, the pots sterilized and the soil autoclaved and reused. New generations of beans were started every week.

3.2.3 Propagation of *B. bassiana*

Isolates of *B. bassiana* were obtained and propagated as described in chapter 2.

3.2.4 Cabbage Looper Rearing

Sixty pupae of cabbage looper, originating from a colony established in 1991 at the University of Alberta, were randomly selected from a group of ≥ 80 pupae, sterilized in a 10% bleach solution and rinsed with sterile deionized water. After passively drying within folded sterile paper towels, the pupae were deposited into large, sterile 4L glass jars with autoclaved white paper towel lids secured in place with an elastic band. Jars of pupae were placed on a shaded counter top for 1.5 - 2 weeks at 23±2°C, 60±5%RH, 16:8 L:D, and adult moths were allowed to emerge. Adult moths were provided with one10 x 30cm autoclaved strip of unbleached brown paper towel for oviposition taped to the inside of the paper towel lid. In addition, all adult moths were provided with four 10mL sterile glass test tubes of 10% sterile honey-water *ad libitum*, capped with sterile cotton batting, to allow capillary movement of honey-water into the cotton. Adult moths were allowed to mate at 23±2°C, 60±5%RH, 16:8 L:D, and the brown paper oviposition strips were removed every two days. Strips were sterilized in a 1% bleach solution, rinsed with a 10% sodium thiosulphate neutralizer solution and then rinsed with sterile, deionized water. After drying within a laminar-flow biocontainment cabinet, the oviposition strips were sliced into 5 x 2cm pieces and 6 pieces were placed into 500mL paper and wax Sweet Heart® (Solo® Cup Company, Highland Park, Illinois, U.S.A.) cups with opaque plastic snap-on lids. All

cups were incubated at 28±2°C, 100%RH, 16:8 L:D. Larvae were fed a 1±0.5 x 3cm block of meridic (artificial) lepidopteran diet cut with a sterile scalpel *ad libitum* for the duration of their development, either to 4th instar for bioassays, or to adult instar for mating. Droppings were removed with a sterile wooden applicator stick each time larvae received a new block of diet. Fourth-instar larvae were divided into groups of 12 per cup.

3.2.4.1 Meridic Lepidopteran Diet

A meridic (artificial) lepidopteran diet was prepared after Tanada and Chang (1968), later modified by Keddie and Volkman (1985). All diet ingredients were manipulated aseptically. After blending at boiling point and cooling to 50°C, the molten diet was poured into sterile Pyrex® 10cm glass dishes. After solidification, all dishes were sealed with masking tape, labelled, and refrigerated at 4°C.

3.2.5 *Podisus maculiventris* Rearing

Eggs of *P. maculiventris*, originating from a colony established in 1998 at the University of Alberta, were harvested from paper towel substrates inside laboratory rearing jars and placed into sterile, 500mL mason-style jars. All jars were covered with autoclaved white paper towel lids secured in place with an elastic band. Nymphal instars 2 - 5 were provided cabbage looper larvae *ad libitum* every 2 days. As nymphs grew older, larger larvae were provided. Nymphs and adults were also provided with a 5 x 5cm piece of cabbage and a clean, crumpled piece of paper towel every 2 days. In preparation for bioassays,

three generations of *P. maculiventris* were maintained, started weekly. Every 2 weeks, healthy male and female adults were paired, and 3 - 4 pairs were placed into each of 10 new jars for holding and mating, for a total of ≥30 pairs for the whole-plant tritrophic bioassays.

3.2.6 Buffers

Phosphate buffers were used for conidial storage and application.

Stock buffer solutions were prepared and stored at 4°C as per chapter 2.

Working buffer solutions, prepared as required, included: 1) phosphate buffer only (pH 7, 0.1M) (see chapter 2); and 2) phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 (see chapter 2).

3.2.7 Propidium Iodide Solutions

A propidium-iodide solution, refrigerated in a foil-wrapped 2000µL microcentrifuge tube, was used for fluorescence-microscopic viability analysis. The propidium-iodide stock solution was prepared every 6 months as per chapter 2.

The propidium-iodide working solutions were prepared the day of the conidial viability assessment as per chapter 2.

3.2.8 Screening of Selected *B. bassiana* Isolates Against Cabbage Looper

Prior to testing *B. bassiana* against cabbage looper and *P. maculiventris*, 6 isolate selection trials were performed testing *B. bassiana* against cabbage

looper. Each bioassay was set up in exactly the same manner as those described in chapter 2 but only 4 Albertan isolates were tested for effects on cabbage looper.

Six isolate selection trials were run testing 4 different Albertan *B. bassiana* isolates, each at $1.0x10^8$ conidia/mL and a carrier-only control. Bioassays were conducted in a completely randomized design with 5 replicate arenas per isolate. Each arena consisted of one 500mL paper and wax Sweet Heart® cup with an opaque plastic-snap on lid containing five 5th instar cabbage looper larvae. All cups were sprayed without lids inside the spray chamber, as per chapter 2. After application of conidia, all cups received one piece of artificial diet followed by a labelled lid; five cups were sprayed per isolate. The experiment was replicated 3 times from production of conidia, insects, and plants, to assessment of viability, all steps of spore preparation, and bioassay, for each of two pairs of *B. bassiana* isolates tested. Bioassays were performed in sequence one hour apart on the same day. Arenas were incubated at 23±2°C, 60±5%RH, 16:8 L:D for 11 days.

Mortality data from the isolate selection trials were subjected to Probit Analysis (for LT_{50}'s only) and Analysis of Variance (ANOVA) using SAS (SAS Institute, Cary, North Carolina) PROBIT and GLM, respectively. One type of ANOVA was run: comparison of all isolates within pooled trials.

Isolate selection trials were pooled for ANOVA only, to increase statistical power. Performance of Alberta *B. bassiana* isolates as biocontrol agents of cabbage looper, were compared within pooled trials via Probit and ANOVA.

3.2.9 Whole-Plant Tritrophic Bioassay

3.2.9.1 Hand-Held Sprayers

Two GreenLeaf® (Greenleaf Products Incorporated, Burnaby, British Columbia) hand-held sprayers were used to deliver conidial suspensions to whole bush bean leaf surfaces. Spray-nozzles were permanently set to deliver a coarse mist to thoroughly coat target surfaces until runoff and to avoid shearing of conidia and clogging of the nozzle. A separate bottle was labelled for each isolate.

3.2.9.2 Preparation for Bioassay

3.2.9.2.1 Solution Preparation, Viability Assays, and Conidal Suspensions

One 2L working solution of phospate buffer (pH 7, 0.01M) + 0.01% Tween-80 was aseptically mixed in an autoclaved 2L Erlenmeyer flask in a laminar-flow biocontainment cabinet. The mouth of the flask was covered with clean parafilm and then the flask was refrigerated.

Samples of conidia from selected *B. bassiana* isolates were obtained and viability of conidia was assessed as per chapter 2.

Dried samples of each selected *B. bassiana* isolate were suspended in phospate buffer (pH 7, 0.01M) + 0.01% Tween-80, and the mixtures vortexed. Conidia-buffer mixtures were then filtered through funnels equipped with 200µm-mesh nylon in-line filters via agitation by metal spatula, into sterile 500mL Pyrex®, thick-walled reagent bottles. Final volume was adjusted with phospate buffer (pH

7, 0.01M) + 0.01% Tween-80, added until an approximate concentration of ≥1.0x10^8 conidia/mL was attained (Rajput, personal communication).

3.2.9.2.2 Set-Up of Bioassay Cages

All cages to be used for the bioassay were checked for structural integrity prior to each bioassay. Each cage comprised a simple 50 x 50 x 100cm wooden frame with a 50x50x1cm plywood bottom lined with a white plastic corrugate. All cages were lined with 80µm-mesh-Nitex® nylon fabric glued in place with silicone sealant. In addition the cages each had a 50 x 100 x 0.5cm Plexiglas door sealed with 1 x 0.5cm thick neoprene adhesive doorjamb weather sealer. All other inside corners of the cages were sealed with silicone sealant (Figure 3-1). All plants previously inserted into bioassay cages were watered and any new growth removed with sharp, sterile scissors.

3.2.9.2.3 Preparation of Corrected Conidial Suspensions

Control solutions consisted only of a 500mL aliquot of the carrier solution phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80.

Concentration of all uncorrected conidial suspensions was assessed with a Reichert Neubauer (Reichert Incorporated, Depew, New York) Bright-Line hemocytometer. After correction for viability, each isolate was mixed with phosphate buffer (pH 7, 0.01M) + 0.01% Tween-80 in clean, designated hand-held sprayers, to provide 500mL suspensions of 1.0x10^7 conidia/mL.

GHA (Emerald Bioagriculture, Butte, Montana), received as an unformulated dry condial sample, served as an internal standard prepared as all

other isolates. Upon receipt, this sample was subcultured once on 0.5x SDAY (without antibiotics) to confirm purity.

3.2.9.3 Initiation of Bioassay

3.2.9.3.1 Bioassay

The bioassay was conducted in a completely randomized design with 5 replicate cages per treatment. For each of 6 treatments where one cage received one treatment, five 4th instar larvae of cabbage looper were placed onto the soil of the caged bush bean plants and allowed to wander. One male-female pair of *P. maculiventris* was then placed in the bottom of designated cages: 1 cage contained cabbage looper only and 1 cage contained cabbage looper and *P. maculiventris* only. Two more cages containing the above combinations of insects but treated with GHA were then prepared, and then 2 more cages as above but treated with an Albertan *B. bassiana* isolate.

Spray bottles were agitated and cages opened at the top corner only. Each suspension was again agitated then sprayed onto the bean leaves until runoff in the following sequence: control → GHA → (sanitize hands) → Alberta isolate; spray intervals and times of exposure were recorded. All cages were incubated at 23±2°C, 60±5%RH, 16:8 L:D for the duration of the bioassay.

3.2.9.3.2 Observations During Bioassay

All cages were checked in the same order in which they were sprayed, starting 24 hours after the bioassay, then every 24 hours for 6 days.

3.2.10 Statistical Analyses

Mortality data from whole-plant bioassays were subjected to Probit Analysis (for LT_{50}'s only) and Analysis of Variance (ANOVA) using SAS (SAS Institute, Cary, North Carolina) PROBIT and GLM, respectively. One type of ANOVA was run: a comparison of all isolates within pooled trials.

Tritrophic trials were pooled for ANOVA only, to increase statistical power. Performance of components of this tritrophic system, including *B. bassiana* and/or *P. maculiventris* as biocontrol agents of cabbage looper, were compared within pooled trials via Probit and ANOVA.

3.3 Results

3.3.1 Screening of Selected *B. bassiana* Isolates for Effects on Cabbage Looper

3.3.1.1 Probit Analysis and Analysis of Variance

All isolates used were tested against each other. A total of 6 isolate-selection trials were performed and the results were subject to Probit Analysis and Analysis of Variance. All trials analyzed had control mortalities of $\leq$5%.

3.3.1.2 Results of Isolate-Selection Trials

Isolates 3, 35, 7, and 25 were highly lethal towards cabbage looper for all pooled isolate selection trials (Table 3-1, Figures 3-2 – 3-4). There were no significant differences between the cumulative mortalities of cabbage looper

caused by these isolates (Table 3-2). Isolate 3 performed better than GHA within all non-pooled and pooled screening trials with WFT, and evaluation trials with WFT (Tables 2-1 - 2-10). Therefore, isolate 3 was selected from the group of isolates used in this experiment.

3.3.2 Evaluation of Selected Strains

3.3.2.1 Evaluation of Virulence of *B. bassiana* Isolates for Cabbage Looper, in Conjunction with Efficacy of Predation on Cabbage Looper by *P. maculiventris*

All isolates used were tested against a control group (carrier only), as well as an internal standard, GHA. A total of 3 tritrophic trials were performed, and the results were subject to Probit Analysis and Analysis of Variance. All trials analyzed had control mortalities of $\leq$5%.

3.3.2.1.1 Performance of Treatments Administered to Cabbage Looper

Isolate 3 and GHA had LT_{50}'s greater than 5 days, thus these isolates required the greatest amount of time to kill 50% of cabbage loopers. All treatments using *P. maculiventris* had LT_{50}'s less than 3 days, thus as a group, all treatments using *P. maculiventris* required the least amount of time to kill cabbage looper (Table 3-3).

Within these tritrophic trials, isolate GHA performed poorly, the time responses varying among tritrophic trials (Table 3-3); these extreme results for GHA are an indicator of the inconsistent effect that this isolate may have on cabbage looper (Table 3-4).

Cumulative mortality of cabbage looper was greatest where *P. maculiventris* was used, whether in conjunction with *B. bassiana* or not, followed by isolate 3 alone, then GHA alone (Table 3-4). Bush bean plants were protected most effectively where *P. maculiventris* was used with the least feeding damage occurring on plants where *P. maculiventris* was used in conjunction with *B. bassiana*, followed by *P. maculiventris* alone, isolate 3 alone, then GHA alone (Figures 3-5 - 3-7 and 3-9 – 3-15). The amount of cabbage looper feeding occurring under different treatments within pooled tritrophic trials differed significantly (Table 3-7).

3.3.2.2 Evaluation of Virulence of *B. bassiana* Isolates for *P. maculiventris*

All trials analyzed had control mortalities of ≤5%.

3.3.2.2.1 Detrimental Effects of Isolates Administered to *P. maculiventris*

The GHA had an LT_{50} greater than 7 days, in 2 of 3 trials, thus GHA may have killed 50% of *P. maculiventris* if given more time than that available for the bioassay (Table 3-5).

Within these tritrophic trials, isolate GHA performed poorly, the time responses varying among tritrophic trials (Table 3-5); these extreme results for GHA are an indicator of the inconsistent effect that this isolate may have on *P. maculiventris* (Table 3-6).

Mortality of *P. maculiventris* did not occur where isolate 3 was used; cumulative mortality occurred only where GHA was used, and was of no significance (Table 3-6).

3.4 Discussion

3.4.1 *Beauveria bassiana* Screening and Evaluation of Results

To ensure that the fungus was indeed killing cabbage looper, trials with control mortalities of 5% or less and those whose cadavers produced *B. bassiana* after surface sterilization and incubation on selective media were retained for analysis (Rajput, personal communication). Only conidial samples with the highest possible viabilities were used (Tables 3-8 and 3-9).

Cabbage looper was decimated by *B. bassiana* mycosis whereas almost all *P. maculiventris* were able to escape mycosis. Hemipterans differ from lepidopterans in cuticular structure, metabolism, development and behaviour (Gullan and Cranston, 2005). All, or a combination, of these differences may have caused the disparity in incidence of infection that was observed. *Podisus maculiventris* adults were observed grooming and hiding in protected spaces while stalking; their bodies are held high from the ground and are well shielded by tough, leathery forewings and a heavily sclerotized cuticle. Conidial contamination is more likely to occur via contact of the tarsi with a contaminated substrate and not through bodily contact with the substrate or aerial contamination. Cabbage looper larvae were not observed grooming and were not feeding in protected spaces; their bodies are not held high from the ground and have a relatively flexible, unsclerotized cuticle. Conidial contamination is more likely to occur via contact of the whole body with the substrate and aerial contamination.

Podisus maculiventris was observed hiding and stalking in secluded areas under leaves and other shaded, secure areas. These secluded areas must be

noted by the agricultural producer and protected from *B. bassiana* contamination, by using appropriate application measures wherever *P. maculiventris* is used as a biocontrol agent. If cabbage looper larvae escape *B. bassiana* in these protected spaces, they will undoubtedly be destroyed by *P. maculiventris*. In epigeal habitats, *B. bassiana* is exposed to limiting environmental factors (Jaronski and Goettel, 1997). Persistence of *B. bassiana* on the phylloplane is affected by morphology of the canopy, location within the canopy, and conidial formulation (Jaronski and Goettel, 1997). A major limiting factor is sunlight (UV), which causes heavy conidial mortality on the phylloplane, if protection from UV penetration is not used; also rainfall/watering, washes conidia from the phylloplane. Conidia applied to the top 10 cm of soil show a gradual decrease in colony-forming-units (CFU) per gram, 4 – 8 weeks post-application, but stabilization has been observed after 20 days (Jaronski and Goettel, 1997). In addition, soil microflora can have negative, fungistatic effects on *B. bassiana* (Jaronski and Goettel, 1997). *Podisus maculiventris* adults may actually avoid contact with *B. bassiana* when it is freshly applied and at its highest virulence and viability through their hiding and stalking behaviour. Thus, *P. maculiventris* may take advantage of hiding places and limiting environmental factors, especially if *B. bassiana* affects them in a limited manner, as they seek and destroy cabbage loopers.

Within holometabolous insect orders, development typically includes a distinct group of similar larval instars, a quiescent (pupal) stage and a distinct adult stage. Differences between adult and larval mortality caused by entomopathogenic fungi occur as a result of differences in cuticular structure, metabolism and behaviour. Also larvae can decontaminate themselves if they moult before the infection process starts, but adults cannot (Donegan and

Lighthart, 1989). All cabbage looper larvae moulted once from 4th instar to 5th instar but almost all larvae treated with *B. bassiana* showed signs and symptoms of mycosis and these larvae eventually died; this indicates that these larvae may have been re-exposed to *B. bassiana* after shedding contaminated cuticle, and that enough viable conidia remained to kill these larvae. Had the length of the bioassay not been limited to 6 days, *P. maculiventris* may have eventually succumbed to mycosis as well. Whatever the outcome for *P. maculiventris*, adults remained alive long enough to destroy nearly all the cabbage looper larvae within their range, and many copulated in a minor follow-up study by Keddie *et al.* (data not shown), then laid eggs, which later hatched to produce healthy nymphs.

Cabbage looper treated with *B. bassiana* fed more slowly in comparison to controls, and caused much less feeding damage before succumbing to mycosis. Where *P. maculiventris* was employed, feeding damage by cabbage looper was reduced further, slightly more so if *B. bassiana* was included.

Podisus maculiventris used with *B. bassiana* prevented feeding damage more effctively in comparison to controls. This may be an indication of synergism, avoidance of infection caused by *B. bassiana* through selection of healthy, suitable prey where *B. bassiana* has been used. *Podisus maculiventris* appeared to demonstrate selective behaviour. During the tritrophic bioassays, they did not feed on cabbage looper larvae showing acute signs of mycosis: yellow coloration, wasting, yellow frass, pigmentation by oosporein (Figures 3-3 and 3-8). Cabbage loopers, which showed an immune response to *B. bassiana* penetration, such as melanotic spots on the cuticle, were destroyed by *P. maculiventris* before they showed any acute signs of mycosis. These results are

incentive to study the selective behaviour of *P. maculiventris* in greater detail concerning their choice between unsuitable and suitable prey items.

Grasshoppers and flies are able to fight fungal infections by raising their body temperature above that optimal for fungal growth by basking in the sun (Jaronski and Goettel, 1997). It is not known whether *P. maculiventris* is able to thermoregulate in such a manner but it would be advantageous to examine the possibility.

3.5 Summary

Isolate 3 performed significantly better than GHA, throughout 3 tritrophic trials when *P. maculiventris* was not used, destroying cabbage loopers. The performance of both isolates was eclipsed by the high efficiency of *P. maculiventris* whether used in conjunction with *B. bassiana* or not. Bush beans were most protected where *P. maculiventris* was used with *B. bassiana*, followed by *P. maculiventris* alone then *B. bassiana* alone. *Podisus maculiventris* treated with GHA experienced limited mortality whereas isolate 3 caused no such mortality. Under these experimental conditions, isolate 3 performed better than GHA in that it destroyed more cabbage loopers more rapidly *and* spared *P. maculiventris*. Thus the systems tested in this project may be compatible for use in integrated pest management programs, and should be further studied, through more rigorous experimentation.

3.6 Tables and Figures

Table 3-1. Performance of Alberta *B. bassiana* isolates tested on cabbage looper, among isolate selection trials (LT_{50} = Days).

Isolate Selection Trial	Isolate	LT_{50}
1a	3	8.03
	35	7.66
1b	3	5.67
	35	14.37
1c	3	6.67
	35	9.66
2a	7	7.70
	25	6.26
2b	7	8.03
	25	8.17
2c	7	6.37
	25	7.47

Table 3-2. Variability of cumulative mortality (%) of cabbage looper caused by Alberta *B. bassiana* isolates, within pooled isolate selection trials (Tukey Means Test, $\alpha = 0.05$, N = 3); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Isolate
90.67 a	9.71	3
85.33 a	9.71	25
81.33 a	9.71	7
56.83 a	9.71	35

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Isolate	3	2.38	0.1459

Table 3-3. Performance of *B. bassiana* isolates and *P. maculiventris* tested on cabbage looper, among tritrophic trials (LT_{50} = Days).

Tritrophic Trial	Treatment	LT_{50}
1	Podisus	1.73
	GHA	6.23
	Podisus &GHA	1.56
	3	6.23
	Podisus & 3	1.49
2	Podisus	1.45
	GHA	2.39E+41
	Podisus &GHA	2.71
	3	5.34
	Podisus & 3	2.20
3	Podisus	0.99
	GHA	21.93
	Podisus &GHA	1.16
	3	6.04
	Podisus & 3	0.96

Table 3-4. Variability of cumulative mortality (%) of cabbage looper caused by *B. bassiana* isolates and *P. maculiventris*, within pooled tritrophic trials (Tukey Means Test, $\alpha = 0.05$, N = 3); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Treatment
95.94 a	7.47	3 & Podisus
95.89 a	7.47	GHA & Podisus
91.89 a	7.47	Podisus
48.61 b	7.47	3
12.39 c	7.47	GHA

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Isolate	4	25.06	<0.0001

Table 3-5. Performance of *B. bassiana* isolates tested on *P. maculiventris*, among tritrophic trials (N.M. = no mortality resulting from treatment, LT_{50} = Days).

Tritrophic Trial	Isolate	LT_{50}
1	GHA	7.28
	3	N.M.
2	GHA	4.28E+46
	3	N.M.
3	GHA	N.M.
	3	N.M.

Table 3-6. Variability of cumulative mortality (%) of *P. maculiventris* caused by *B. bassiana* isolates, within pooled tritrophic trials (Tukey Means Test, $\alpha = 0.05$, N = 3); trials with the same letter were not significantly different.

Mean Cumulative Mortality (%)	±SE	Isolate
10.00 a	4.08	GHA
0.00 a	4.08	3

ANOVA Table (Type III S.S.)

Source	DF	F-Value	P-Value
Isolate	1	3.00	0.1583

Table 3-7. Comparison of amount of cabbage looper feeding occurring under different treatments within pooled tritrophic trials (ordinal scale data, non-parametric statistics; $\alpha = 0.05$).

Treatment	Count	DF	Rank Sum
None	15	5	1222.000
P. maculiventris	15	5	526.000
GHA	15	5	880.000
P. maculiventris and GHA	15	5	380.000
Isolate 3	15	5	769.000
P. maculiventris and Isolate 3	15	5	318.000
Kruskal-Wallis Test Statistic = 62.681		P-Value = 0.000	

Table 3-8. Viability of *B. bassiana* conidia used, and cabbage looper control mortality recorded among isolate selection trials.

Isolate Selection Trial	Isolate	Viablity (= % Live Conidia)
1a,b,c	3	99.8
1a,b,c	35	100.0
2a,b,c	7	99.9
2a,b,c	25	93.6

Isolate Selection Trial	Percentage Control Mortality
1a	0.0
1b	0.0
1c	4.0
2a	0.0
2b	0.0
2c	0.0

Table 3-9. Viability of *B. bassiana* conidia used, and insect control mortality recorded among tritrophic trials.

Tritrophic Trial	Isolate	Viablity (= % Live Conidia)
1	3	99.0
1	GHA	96.8
2	3	99.0
2	GHA	96.8
3	3	99.0
3	GHA	96.8

Tritrophic Trial	Insect	Percentage Control Mortality
1	*T. ni*	0.0
2	*T. ni*	4.0
3	*T. ni*	0.0
1	*P. maculiventris*	0.0
2	*P. maculiventris*	0.0
3	*P. maculiventris*	0.0

Figure 3-1. Insect bioassay cage with 2 pots of 18-day-old bush beans.

Figure 3-2. Unusual blistering of cuticle of 4th instar cabbage looper attempting to moult after application of isolate 7.

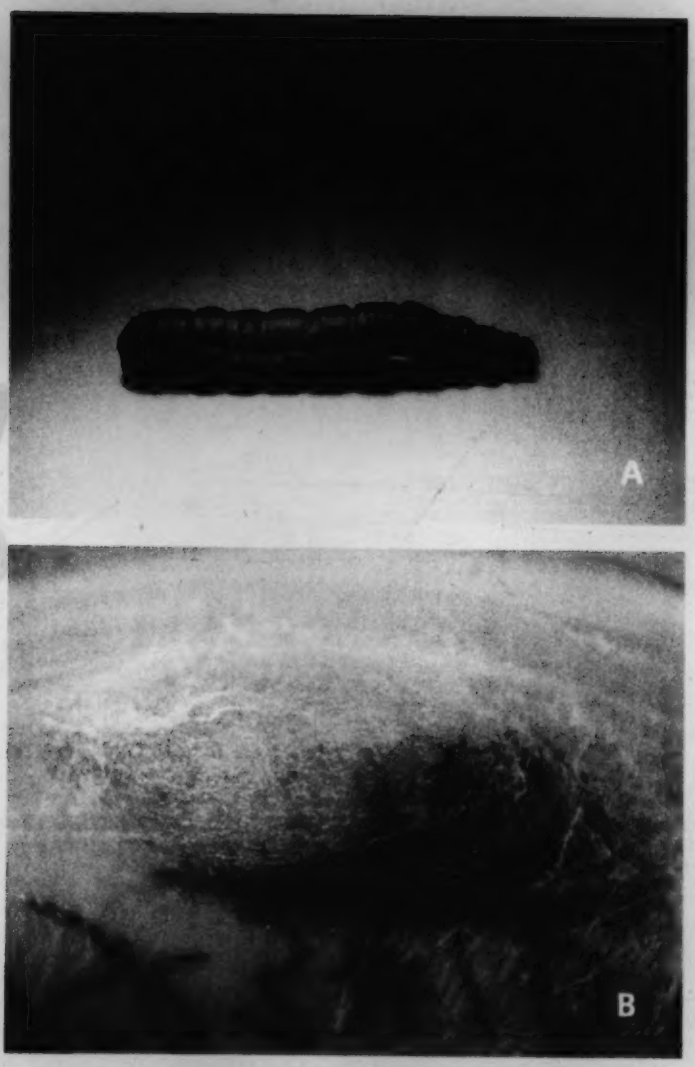

Figure 3-3. Cabbage looper 5th instar (A) and prepupa (B) killed by *B bassiana* and coloured by oosporein.

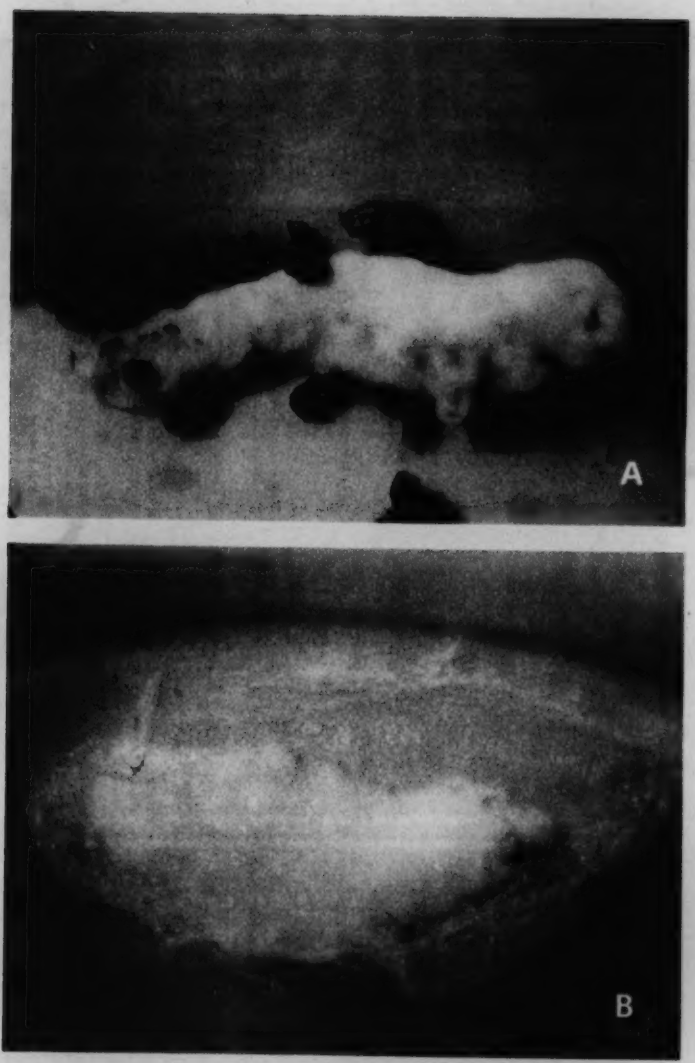

Figure 3-4. Cabbage looper 5th instar (A) and prepupa (B) killed by *B. bassiana* and covered with white conidiophores.

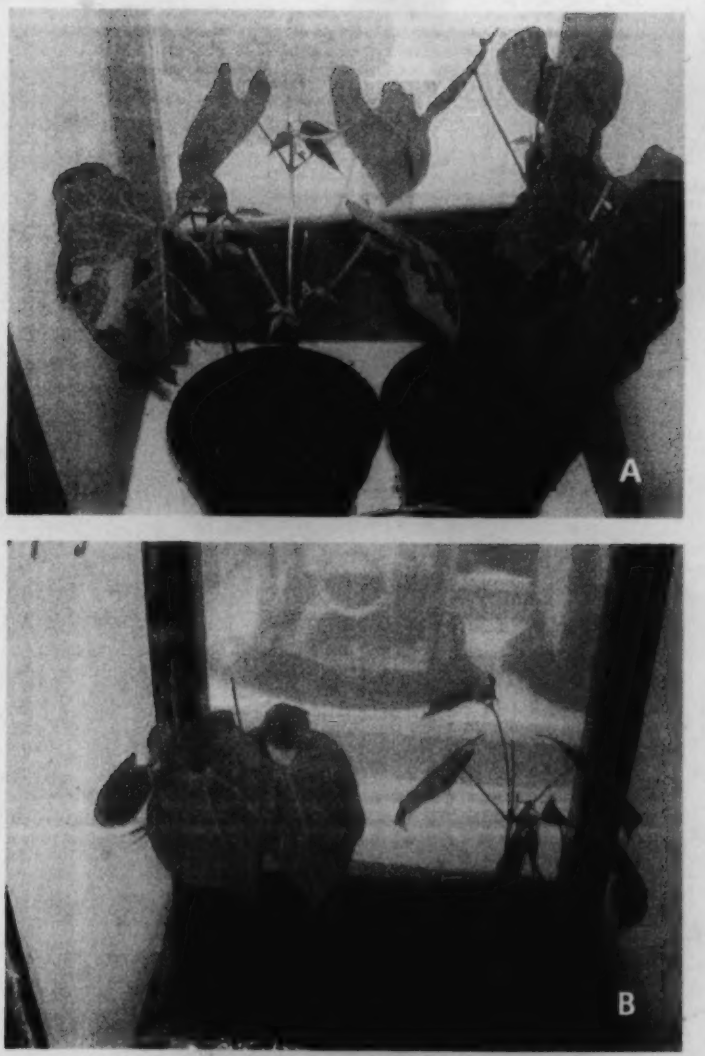

Figure 3-5. Feeding damage to bush bean leaves caused by cabbage looper without any treatment (A) and treated with *P. maculiventris* (B).

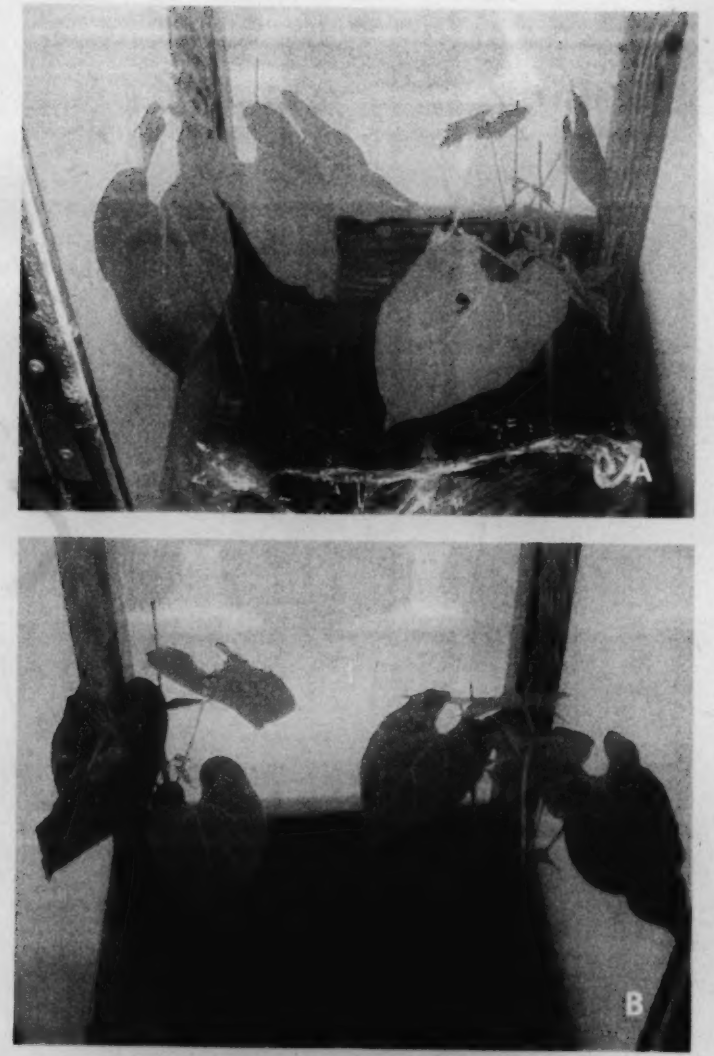

Figure 3-6. Feeding damage to bush bean leaves caused by cabbage looper treated with isolate GHA (A) and treated with isolate GHA and *P. maculiventris* (B).

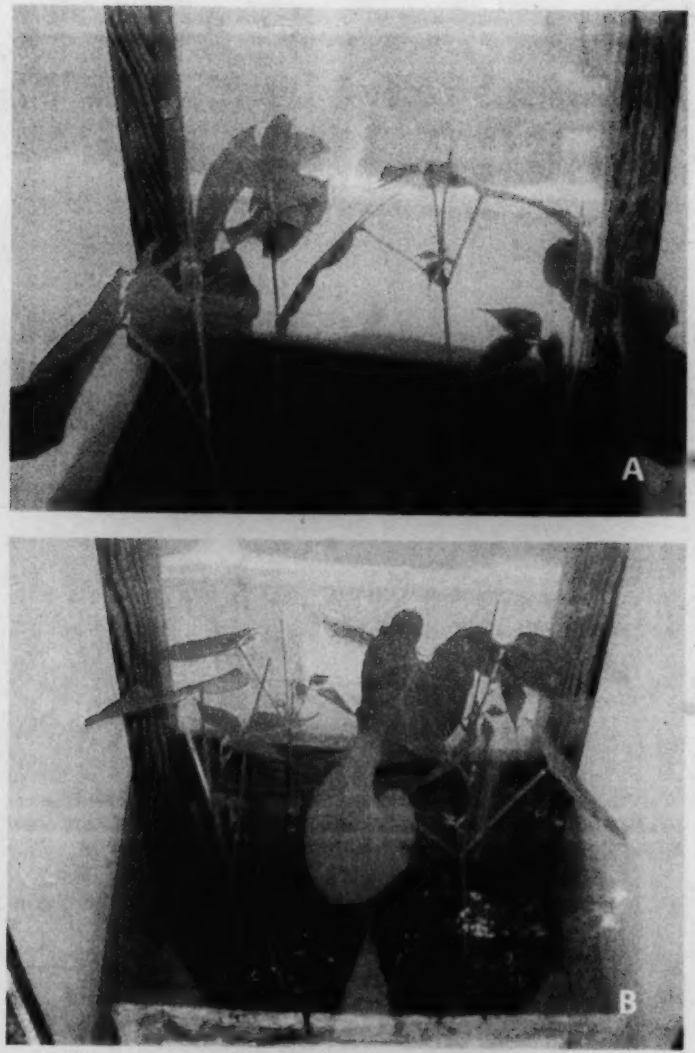

Figure 3-7. Feeding damage to bush bean leaves caused by cabbage looper treated with isolate 3 (A) and treated with isolate 3 and *P. maculiventris* (B).

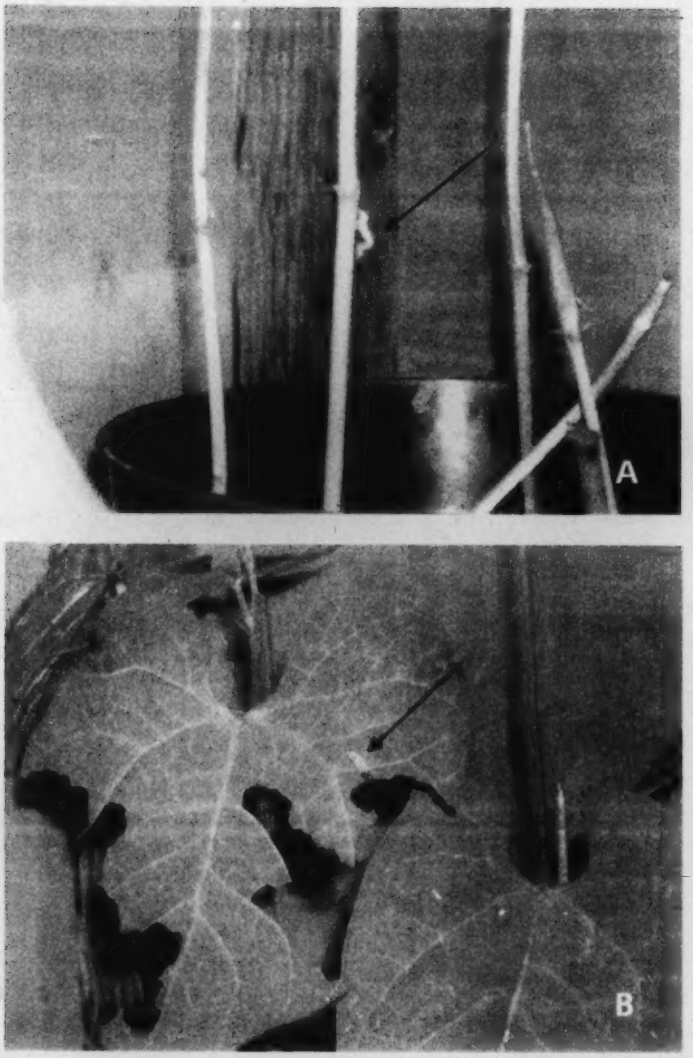

Figure 3-8. Cabbage looper showing acute signs of *B bassiana* mycosis: yellow coloration and wasting caused by isolate 3 (A), and wasting caused by isolate GHA (B).

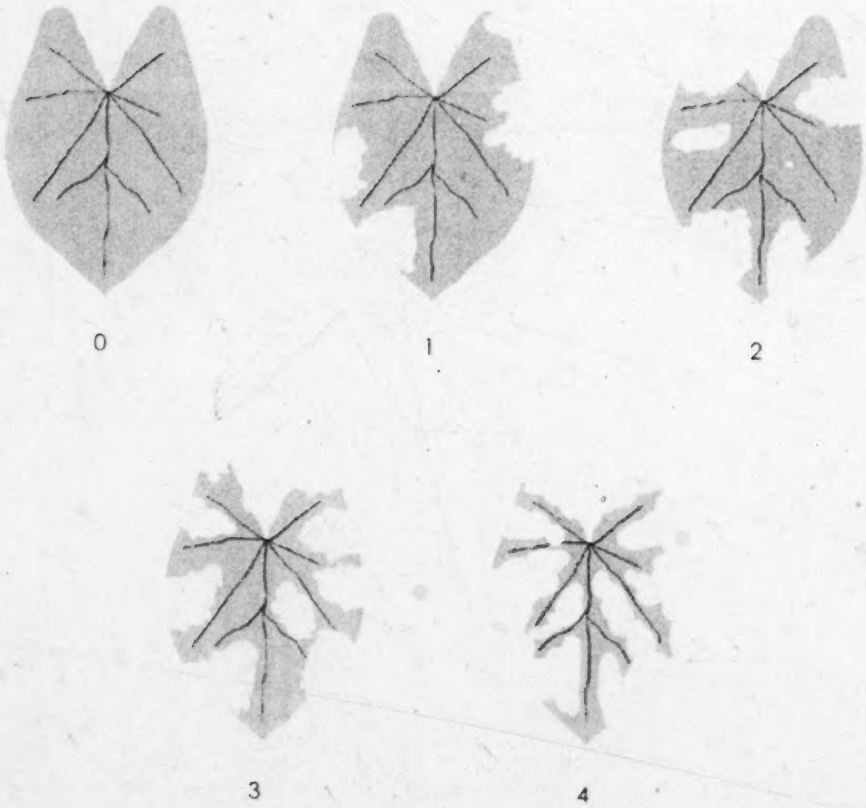

Figure 3-9. Degrees of feeding damage caused by cabbage looper (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization; average feeding damage scores were obtained for each treatment, and rounded to the nearest whole number).

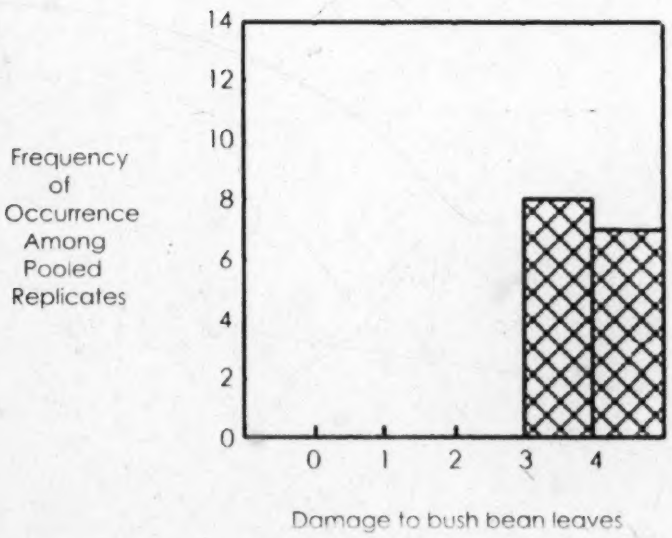

Figure 3-10. Feeding damage to bush bean leaves, caused by cabbage looper without any treatment (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

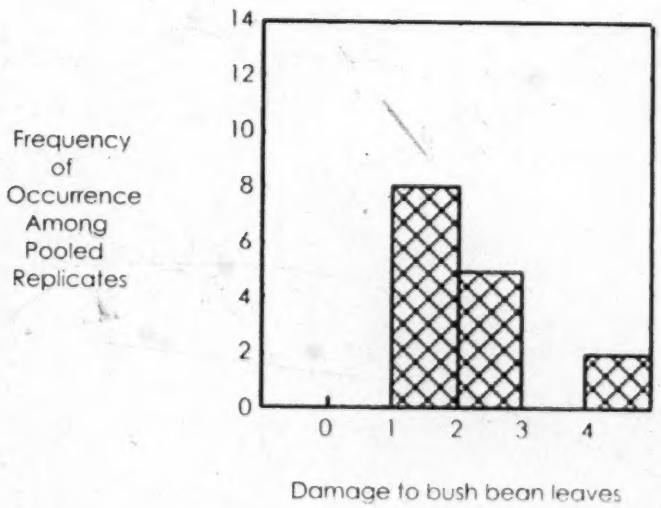

Figure 3-11. Feeding damage to bush bean leaves, caused by cabbage looper treated with isolate GHA only (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

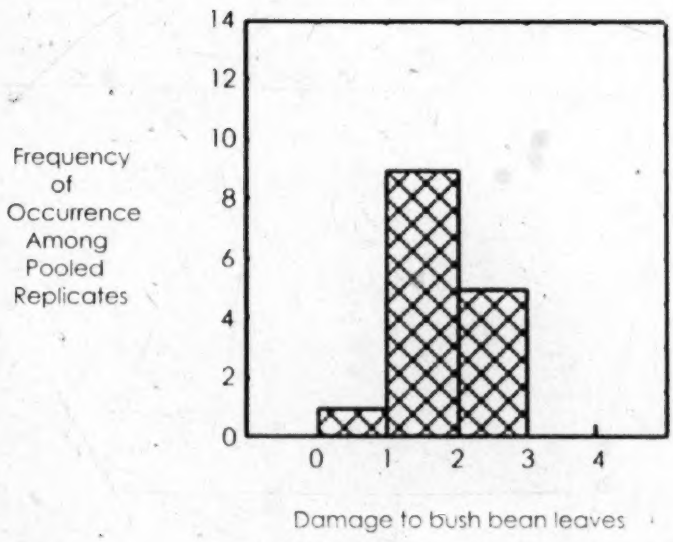

Figure 3-12. Feeding damage to bush bean leaves, caused by cabbage looper treated with isolate 3 only (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

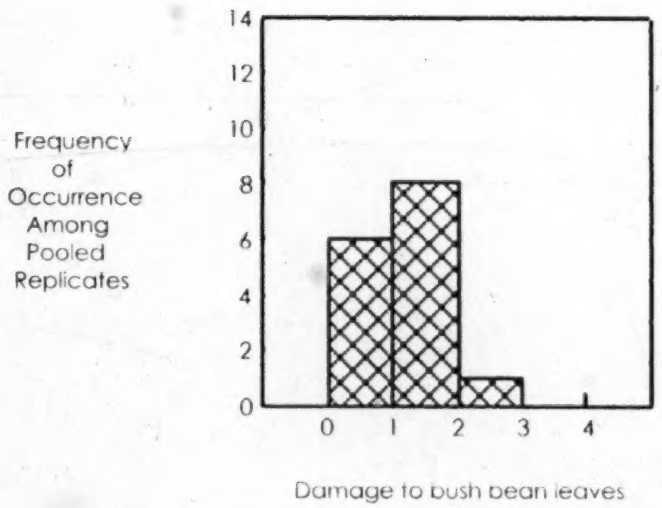

Figure 3-13. Feeding damage to bush bean leaves, caused by cabbage looper treated with *P. maculiventris* only (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

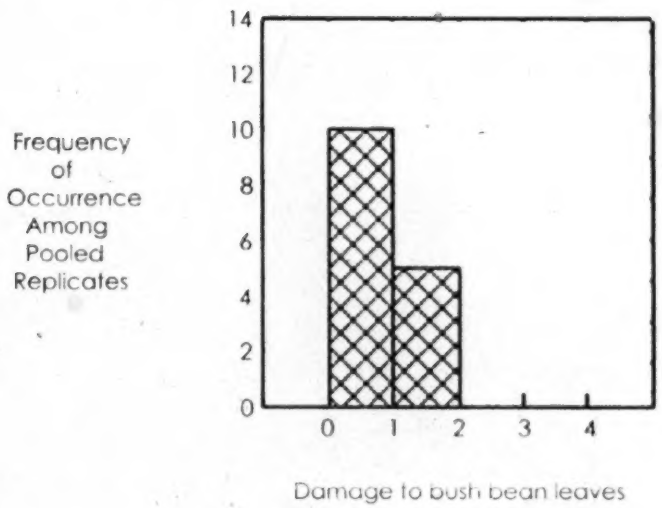

Figure 3-14. Feeding damage to bush bean leaves, caused by cabbage looper treated with *P. maculiventris* and isolate GHA (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

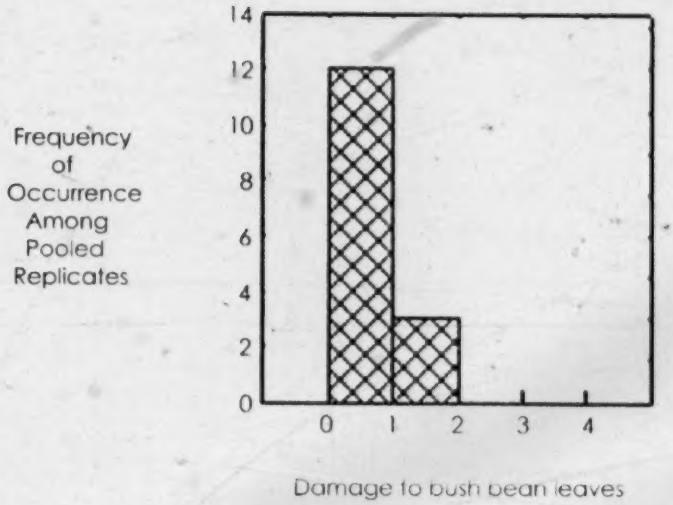

Figure 3-15. Feeding damage to bush bean leaves, caused by cabbage looper treated with *P. maculiventris* and isolate 3 (0 = no feeding, 1 = little feeding, 2 = feeding, 3 = excessive feeding, 4 = skeletonization).

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Chapter 4. General Discussion and Conclusions.

4.1 The Need for Environmentally Friendly Insecticides

Modern agricultural practices make use of specially selected cultivars of crop plants which, if grown in conjunction with pesticides, fertilizers and controlled irrigation, produce far greater yields than traditional cultivars and methods (Rosset *et al.*, 2001). These new agricultural practices affect surrounding environments with severe ecological ramifications in time and space. Environmental costs have been accumulating steadily, ranging from pollution of groundwater to loss and deterioration of ecosystems (Lacey and Goettel, 1995). Insect populations have developed resistance to many pesticides (Lacey and Goettel, 1995). Subsequent pest resurgences and secondary pest outbreaks have occurred due to elimination of natural enemies (Lacey and Goettel, 1995). The end result is ever-increasing difficulty and spending to reach production rates comparable to those of the past (Rosset *et al.*, 2001).

Some pesticide residues remain in the environment, even though these products are no longer used, and are found on food grown in contaminated soil and in fish living in contaminated water (Ghandi and Snedker, 1999). Residue levels found in food are usually below tolerance levels set by the Environmental Protection Agency (EPA), but some pesticides have been found in food for which no tolerances have been set. Despite setting specific regulations for tolerable pesticide residues, a small percentage of domestic and imported foods violate the residue tolerances set by the EPA (Ghandi and Snedker, 1999).

Integrated pest management, defined as "a strategy of pest containment that seeks to maximize the effectiveness of biological and cultural control factors,

using chemical controls only as needed and with a minimum environmental disturbance", is a viable solution to the problem of controlling pest insects which attack agricultural and horticultural crops (Luna and House, 1990). Entomopathogenic bacteria, fungi and viruses are ideal for integrated pest management programs because they are relatively safe to use and have a narrower spectrum of activity than chemical insecticides (Lacey and Goettel, 1995). Potential antagonistic effects can be limited through careful selection of entomopathogens which have a greater effect on pest insects than on their predators or parasitoids (Luna and House, 1990; Lacey and Goettel, 1995).

4.2 Benefits of Using Microbial Insecticides Alone or in Conjunction with Beneficial Insects Instead of Chemical Insecticides

Greenhouses, artificial habitats situated within the environment, can be summarized from a metapopulation perspective as empty artificial patches amongst naturally occurring patches. Metapopulation dynamics operate at two levels, within and amongst patches, where the degree of recolonization increases with number of empty patches prone to recolonization and with number of occupied patches able to provide colonizers (Begon *et al.*, 1996). Extinction increases with number of patches prone to extinction; therefore as long as the intrinsic rate of recolonization exceeds the intrinsic rate of extinction, the total metapopulation will reach a stable equilibrium (Begon *et al.*, 1996). A metapopulation persists as a result of random extinctions and recolonizations, the local populations themselves are unstable. Larger patches also maintain the metapopulation by supporting larger local populations which experience lower rates of extinction and recolonization (Begon *et al.*, 1996).

Patches also vary in quality, with high-quality patches as sources (donors) and low-quality patches as sinks (receivers) (Begon *et al.*, 1996). In source patches birth rates exceed death rates and the reverse is true for sink patches. Therefore source populations support sink populations allowing the metapopulation to persist (Begon *et al.*, 1996).

Sink patches may be divided into true sink patches and pseudo-sink patches. Deaths exceed births in a true sink patch but immigrants exceed emigrants when the metapopulation is at equilibrium. In a pseudo-sink patch, deaths exceed births at the metapopulation equilibrium but only through the occurrence of density-dependent decreases in birth rate or increases in mortality (Begon *et al.*, 1996).

Greenhouses are sterile, artificial patches which are prone to colonization and recolonization by pest insects especially if surrounded by occupied patches that are able to provide colonizers. When a local metapopulation of pest insects is at equilibrium, greenhouses are excellent patches for colonization. Once colonized, a greenhouse becomes a superb source patch where birth rates exceed death rates. Use of pesticides destroys all pests and predators in turn allowing for recolonization from other source patches. The greenhouse quickly becomes an unoccupied patch remaining prone to recolonization, especially if pest insects have become or are in the process of becoming resistant to the chemical insecticides used. As with use of chemical insecticides, use of entomopathogenic fungi such as *B. bassiana* can change a greenhouse from a source patch into a sink patch. Entomopathogenic fungi have been shown to be relatively specific and isolates have been identified which attack pest insects and spare beneficial insects. Greenhouses employing *B. bassiana* as an inundative insecticide effectively control infestations of pest insects; the sink-status

of the greenhouse requires direct human intervention by application of *B. bassiana*. Also, infected insects can leave the greenhouse patch and infect surrounding patches. The greenhouse can also be maintained as a pseudo-sink if beneficial insects are able to escape *B. bassiana* infection either through their behavioural or physiological attributes, which is unattainable if chemical pesticides are used. Beneficial insects can be permanently employed by routine enhancement of their populations with samples obtained from commercial or natural sources; the pseudo-sink status of the greenhouse also requires direct human intervention. To prevent the greenhouse from becoming a sink for beneficial insects, alternate food supplies can be used to help many types of beneficial insects survive periods of low pest insect density.

Preventing primary and secondary pest outbreaks may be as simple as including greenhouses in the local metapopulation and by maintaining them as sink and pseudo-sink patches, by using entomopathogenic fungi such as *B. bassiana* alone or in conjunction with beneficial insects.

4.3 Goals of This Project

I have screened native Alberta isolates of *Beauveria bassiana* (Balsamo) Vuillemin for efficacy against western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) and tested selected isolates for positive and negative effects in a tritrophic system including cabbage looper, *Trichoplusia ni* (Hübner), *B. bassiana* and a natural preadator and biocontrol agent of cabbage looper, the spined soldier bug *Podisus maculiventris* (Say).

4.3.1 Control of Western Flower Thrips with Native Alberta *B. bassiana* Isolates

Isolates 3, 35, 7, and 25 were more virulent than GHA in 3 replicate evaluation trials, whereas isolate 37 was moderately more virulent than GHA, and isolates 15, 19, and 11 less virulent than or similarly virulent to GHA. The isolates performing better than GHA in this study have the potential to be developed into microbial control agents for WFT, and should be further studied through more rigorous experimentation.

4.3.2 Control of Cabbage Looper with Native Alberta *B. bassiana* Isolates, in Conjunction with *P. maculiventris*

Isolate 3 performed significantly better than GHA, throughout 3 tritrophic trials when *P. maculiventris* was not used, destroying cabbage loopers. The performance of both isolates was eclipsed by the high efficiency of *P. maculiventris* whether used in conjunction with *B. bassiana* or not. Bush beans were most protected where *P. maculiventris* was used with *B. bassiana*, followed by *P. maculiventris* alone then *B. bassiana* alone. *Podisus maculiventris* treated with GHA experienced limited mortality whereas isolate 3 caused no such mortality. Isolate 3 performed better than GHA in that it destroyed more cabbage loopers more rapidly *and* spared *P. maculiventris*. Thus the systems tested in this project may be compatible for use in integrated pest management programs, and should be further studied, through more rigorous experimentation.

4.4 The Importance of Standardizing Experimental Procedures

The difference in performance among and within isolates tested both in this project and at the ARC show that standardization is a requirement for laboratories screening isolates of *B. bassiana*, to reliably compare results obtained from different laboratories. Abiotic factors including light, humidity and temperature regimes must be standardized among laboratories performing such bioassays. Production of *B. bassiana* and test subjects, bioassay protocols and equipment used for bioassays, as well as aseptic technique must also be standardized. Biotic factors including *B. bassiana* and test subjects should be standardized for quality. A type culture of *B. bassiana* should be selected as a world standard for all laboratories, instead of using a highly variable, mass-produced, commercially available isolate, such as Emerald BioAgriculture's GHA. Such use of a type culture will increase reliability and comparability of results among laboratories. Emerald BioAgriculture's GHA is a proprietary isolate therefore its source and other vital statistics are kept from the public. The number of replicate bioassays must also be standardized among laboratories, to establish a reliable data set from which to draw conclusions about the bioassays and compare the results among laboratories.

4.5 The Importance of Screening Isolates Through Replicated Bioassays

Both inter- and intra-isolate variability affect the performance of an isolate in time and space. Therefore isolates must be screened rigourously, i.e. replicated at least three times in a highly standardized fashion, to characterize reliably the virulence of an isolate toward a particular pest or beneficial insect.

Replication of screening bioassays will confirm the status of the isolates tested as either highly or weakly virulent in comparison to a standard, and help to uncover hidden variation within isolates.

4.6 The Need to Extend This Research

Western flower thrips is a major cosmopolitan pest of greenhouse ornamentals and vegetable crops (Pelikan, 1989; Izhevskyi, 1992; Katayama and Tatara, 1994; Gerin *et al.*, 1994; Vacante, 1995; Murphy *et al.*, 1998). Cabbage looper is also a widespread polyphytophagous pest of cultivated crops (Vail *et al.* 1971). Economic damage caused by populations of such insects and pesticide resistance within their populations has resulted in mass usage of chemical insecticides worldwide. Development of new biological control agents for use in integrated pest management programs can help reduce dependence of the agriculture industry on chemical insecticides and thus limit the environmental impact caused by their use.

4.6.1 The Biocontrol Network

The Biocontrol Network (BCN) of Canada was recently constituted with the aim of reducing usage of pesticides in agriculture and forestry while replacing them with natural enemies. The BCN is an NSERC (Natural Sciences and Engineering Research Council of Canada) research network which brings together scientists from a wide range of disciplines. This network of scientists shares its knowledge with stakeholders and regulatory agencies, such as the Pest Management Regulatory Agency, PMRA, and is also responsible for training

students (BCN, 2004). This network will serve as an agent for world wide change, from a chemical insecticide-based agricultural paradigm to one which focuses on biological control of insects, through recruitment and education of new researchers. This network must be allowed to persist further, as time and funding is needed in order for it to make the changes it seeks to accomplish.

4.6.2 The Pest Management Regulatory Agency

The Pest Management Regulatory Agency (PMRA) was constituted to deal with the multiple problems inherent in pest management research. The PMRA attempts to facilitate research and registration processes in such a way as to help keep pest populations under control in a sustainable manner, as well as protect people and the environment from unacceptable risks from use of pest control products. The regulatory process is executed in an open manner, conditionally allowing access to such products for sustainable pest management strategies (PMRA, 2003). Effects of *B. bassiana* on human beings, other animals, our environment and especially natural enemies such as *P. maculiventris*, must be thoroughly researched in order to maintain usefulness, sustainability and stability of integrated pest management programs.

4.6.3 Acceptance of Biological Control Agents Worldwide

Government institutions such as the BCN and PMRA are useful for developing, registering and regulating pesticides in Canada. However greenhouse growers, scientists, the BCN, the PMRA and other regulatory

institutions must be proactive in their approach, seeking and providing funding, and developing incentives for sustained pest management research.

Companies commercializing microbials desire clear, consistent federal regulation of such products in the regulatory framework (Starnes *et al.* 1993). Public education regarding microbial insecticides is vital to their acceptance and widespread use (Starnes *et al.* 1993). Funding for research into selecting and mass-producing marketable strains of *B. bassiana* and effective formulation and application technology is required before any major commercial ventures are to be undertaken (Starnes *et al.* 1993). Also, research is required in order to understand the environmental dynamics associated with the use a microbial, for optimal results (Starnes *et al.* 1993).

4.7 Further Uses for This Research

This research is a stepping stone for registration of the isolates of *B. bassiana* tested herein as microbial insecticides for use in Canada and perhaps other regions of the world. Further research is required to determine the potential impact on the environment associated with use of these isolates. In addition, genetic and metabolic analyses will help developers choose isolates of *B. bassiana* which are appropriately virulent, persistent and environmentally friendly.

Beauveria bassiana is a naturally occurring fungus, which has been recognized for decades as a potential biological control agent. This fungus can be mass produced and marketed to greenhouse growers in a variety of formulations, for instance as Emerald BioAgriculture's (formerly Mycotech Corporation) proprietary strain GHA. *Beauveria bassiana* is dynamic and attacks insects through a variety of mechanisms which evolve with insect populations

and characterize a particular strain's virulence. There is potential far beyond current efforts in Canada to develop this fungus for control of insect pests, to be used as an integral part of an integrated pest management program. After further experimentation and comparison of results, there may be an opportunity to develop isolates tested herein into registered microbial biological control agents for use in specific integrated pest management programs.

Greenhouse growers and government institutions must be proactive in their approach to accessing funds for further research towards developing and registering microbial insecticides such as those tested in this project. Public education is vital to acceptance of their development and use.

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Appendices

The following appendices contain results of tests performed to assess quality of data analyzed during this project (Tables A1-1 – A1-4). The coefficient of determination is an overall indication of accuracy with which a fitted regression function predicts dependence of Y on X (independent variable). It is calculated by obtaining the total sum-of-squares (SS) then obtaining a linear regression SS. If each data point falls exactly on the regression line then the value of the regression SS will be equal to that of the total SS, however this is unlikely. The proportion of the total variation in Y that is explained by the fitted regression is termed the coefficient of determination or r-square, whereby r-square = regression SS / total SS; the r-square values have been obtained using SAS (SAS Institute Cary, North Carolina) GLM. The proportion of the total variation in Y is adequately explained by the fitted regression for all trials performed in this project, with values of ≥ 0.4000 (Fry, personal communication).

Tests for normality typically employed include simple cursory examinations or "eyeballing" of 4 main indices of normality. These indices may be presented as scatterplots, box plots, histograms or probablily plots. These methods are highly subjective, therfore I have also included objective statistical procedures which test a null hypothesis, that the data are normally distributed. These tests include the Shapiro-Wilk, Kolmogoriv-Smirnov, Cramer-Von Mises and Anderson-Darling tests for normality; these tests for normality have been obtained using SAS (SAS Institute Cary, North Carolina) GLM and Interactive Data Analysis. These tests indicate that the data analyzed during this project are normally distributed.

Table A1-1. R-square values and tests for normality for all screening trials (α = 0.05).

Trial	R-Square
1 - 7 Pooled GHA	0.6316
1	0.8317
2	0.8531
3	0.7824
4	0.4933
5	0.8063
6	0.8297
7	0.8704
1,2,3,5,6 Pooled	0.7662
2,3,4,5,6,7 Pooled	0.7255

Tests for Normality

Trial	Shapiro-Wilk	Kolmogoriv-Smirnov	Cramer-von Mises	Anderson-Darling
1	0.7595	>.15	>.25	>.25
2	0.2032	>.15	0.2420	0.2413
3	0.5051	>.15	>.25	>.25
4	0.8512	>.15	>.25	>.25
5	0.3884	>.15	>.25	>.25
6	0.8019	>.15	>.25	>.25
7	0.7570	>.15	>.25	>.25

Table A1-2. R-square values and tests for normality for all evaluation trials (α = 0.05).

Evaluation Trial	R-Square
1a,b,c Pooled GHA	0.6607
1a	0.7932
1b	0.8500
1c	0.7292
1a,b,c Pooled	0.6410
2a,b,c Pooled GHA	0.6347
2a	0.8139
2b	0.8518
2c	0.8033
2a,b,c Pooled	0.7475

	Tests for Normality			
Evaluation Trial	Shapiro-Wilk	Kolmogoriv-Smirnov	Cramer-von Mises	Anderson-Darling
1a	0.9867	>.15	>.25	>.25
1b	0.8595	>.15	>.25	>.25
1c	0.2102	0.1064	0.1241	0.1676
2a	0.8772	>.15	>.25	>.25
2b	0.0974	>.15	0.1891	0.1472
2c	0.7275	>.15	>.25	>.25

Table A1-3. R-square values and tests for normality for all isolate selection trials (α = 0.05).

Isolate Selection Trial	R-Square
1a,b,c,2a,b,c Pooled	0.4711

	Tests for Normality			
Isolate Selection Trial	Shapiro-Wilk	Kolmogoriv-Smimov	Cramer-von Mises	Anderson-Darling
1a,b,c,2a,b,c Pooled	0.2402	0.1353	0.0944	0.1084

Table A1-4. R-square values and tests for normality for all tritrophic trials (α = 0.05).

Tritrophic Trial	R-Square
1,2,3 *T. ni* Pooled	0.9093
1,2,3 *P. maculiventris* Pooled	0.4286

	Tests for Normality			
Tritrophic Trial	Shapiro-Wilk	Kolmogoriv-Smirnov	Cramer-von Mises	Anderson-Darling
1,2,3 *T. ni* Pooled	0.1159	>.15	0.1287	0.0916
1,2,3 *P. maculiventris* Pooled	0.1010	0.0359	0.0155	0.0298